REMARKS

A minor error in claim 244 which was presented in Applicants' September 18, 1995 Preliminary Amendment has been corrected above. Inadvertently, the term "nucleotide" was substituted for "oligo- or polynucleotide." With the proper recitation of "oligo- or polynucleotide" having been restored, claim 244 now properly depends from claim 243, the latter also reciting the "oligo- or polynucleotide." Except for this minor amendment, the claims presented for examination in this application continue to be represented by claims 204-224 and 227-258.

The Rejection Under 35 U.S.C. §103

In their September 18, 1995 Second Preliminary Amendment, Applicants addressed the obviousness rejection set forth in the October 4, 1994 Office Action issued in the parent application (Serial No. 08/046,004, filed on April 9, 1993). In addition to the remarks which appear on pages 14-16 of their September 18, 1995 Second Preliminary Amendment, Applicants would like to present the remarks below.

In the cited Kourilsky document, GB 2 019 408[1] (published on October 31, 1979), the following is disclosed beginning on page 1, line 61, and continuing through page 2, line 6:

> The method of detection according to the invention of the possible presence or of the characterization of a sequence or particular fragment of nucleic acid, notably of a gene, even of the whole nucleic acid in a complex sample of nucleic acids, by contacting the sample, if necessary after prior denaturation of the nucleic acid under study, with a probe comprising a complementary nucleic acid, capable of being hybridized with the nucleic acid sequence or the nucleic acid sought, is characterized in that the reagent or probe used is a probe modified chemically by coupling or for its coupling with an enzyme prior or subsequent to the hybridization reaction, the possible presence of nucleic acid sequence or of the nucleic acid sought being revealable by the action of the thus-transformed hybridization product of the probe and of the sequence or of the nucleic acid sought, on an enzyme substrate.

[1] **Applicants note that a corresponding application to Kourilsky's priority document (FR 7810975) was filed in the U.S. Patent and Trademark Office on April 13, 1979 and it eventually issued on April 8, 1986 as Kourilsky et al., U.S. Patent No. 4,581,333. A copy of the aforementioned '333 patent was previously submitted as Exhibit 52 in Applicants' August 22, 1994 Information Disclosure Statement Under 37 C.F.R. §§1.56 & 1.97-1.98 filed in the parent application.**

For the Examiner's convenience and review, copies of Manning's 1975 and 1977 publications, and Avrameas' 1969 publication are attached to this Supplemental Amendment as Exhibits 1, 2 and 3, respectively.

The 1975 and 1977 Manning publications (Exhibits 1 and 2) are directed to gene mapping and gene enrichment techniques, respectively. As noted in the 1977 Manning publication (Exhibit 2), the radioactive labeled "*E. coli* DNA, coupled with cytochrome *c*-biotin, was prepared as described previously" (citing Manning et al. 1975a [that is, the 1975 Chromosoma paper (Exhibit 1) with some modifications. See 1977 Manning publication (Exhibit 2), page 1365, right column, first full paragraph.

In the 1975 Manning publication (Exhibit 1), the technique for coupling RNA to biotin-labeled cytochrome c involves terminal labeling of the sugar. The hydroxyl groups on the terminal sugar at the 3' end of RNA are oxidized and converted to the dialdehyde which is reactive with an amine. Cytochrome c is useful for Manning's purposes because it is spherical in shape, having fourteen (14) amine groups, one of which can react with the thus-formed dialdehyde and some others of which biotin can be incorporated as a label. Thus, Manning's disclosure is directed to the labeling the terminal sugar of RNA, and it is even questionable whether the terminal ribonucleotide is labeled as a nucleotide because the ribose sugar has been grossly changed by its convertion to a morpholine. What is unquestionable in Manning's disclosures is the lack of phosphate involvement in their labelling technique.

Avrameas (Exhibit 3) disclose the conjugation of enzymes with proteins using glutaraldehyde as the coupling agent. The free amino groups of proteins are said to participate in the cross-linking reaction with glutaraldehyde. See Avrameas (Exhibit 3), page 46, last three lines, through page 47, first two lines. Thus, the Avrameas publication itself is not even concerned with nucleic acid.

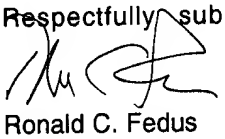
In view of the foregoing remarks, submitted exhibits and their previous remarks presented in their September 18, 1995 Second Preliminary Amendment, Applicants respectfully urge that the previous obviousness rejection under 35 U.S.C. §103 be thoroughly reconsidered before it is applied again to the instant claims.

* * * * * * *

No fee is believed due for this Supplemental Amendment, the fees of $730.00, $220.00 and $506.00 [total of $1,456.00] having been paid with previous filings. In the event that any fee is due, however, The Patent and Trademark Office is hereby authorized to charge the amount any such fee to Deposit Account No. 05-1135, or to credit any overpayment thereto.

Early and favorable action on the claims presented for examination is courteously solicited.

Respectfully submitted,



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A Method for Gene Enrichment Based on the Avidin–Biotin Interaction. Application to the *Drosophila* Ribosomal RNA Genes†

Jerry Manning,* Maria Pellegrini, and Norman Davidson

ABSTRACT: A method of enriching, from the total DNA of an organism, for long DNA strands carrying a particular gene is described. The purified RNA corresponding to the gene is covalently attached to biotin via a cytochrome *c* bridge. This modified RNA is hybridized to the total DNA. Those DNA strands which hybridize are separated from all the other DNA, using the avidin–biotin interaction, by one of two methods. Avidin is covalently attached to submicroscopic polymer spheres; the complexes of avidin spheres with the DNA:RNA–biotin hybrids band in CsCl at a much lower buoyant density than does free DNA. Alternatively, the DNA:RNA–biotin hybrids are isolated by affinity chromatography on an avidin–solid support column. These methods have been used to prepare long single strands of *Drosophila* ribosomal DNA (rDNA) in high yield and 42 to 80% pure.

The preparation of highly enriched fractions of specific DNA segments from the eukaryotic genome is useful in the study of sequence organization and its relation to gene expression. Many interesting problems require that the DNA segments be long, so that the relation of a gene, or other specific sequence, to its flanking sequences can be studied.

In a number of cases, for clustered, reiterated genes such as the rRNA genes of *Xenopus* and the histone genes of the sea urchin (Wallace and Birnstiel, 1966; Brown and Weber, 1968; Kedes and Birnstiel, 1971; Brown et al., 1971) enrichment is possible by buoyant banding. These procedures require that the DNA segment which is to be selected have a different base composition than the average for the genomic DNA and/or that it contain a repeated sequence with a special affinity for a heavy metal ion or a dye, thus shifting the buoyant density.

In principle, if the RNA coded for by the gene of interest is available in reasonable purity and in sufficient quantity, the straightforward way to enrich for the gene is to make an RNA:DNA hybrid and to devise a procedure in which the RNA:DNA hybrid is separated from all the unhybridized DNA. Several interesting experimental realizations of this general strategy have been described (Shih and Martin, 1973; Anderson and Schimke, 1976; Miller et al., 1974). Because of the recent discovery of the phenomenon of R-loop formation (Thomas et al., 1976), effective techniques for the separation of RNA:DNA hybrids from pure DNA will be useful, at least in some cases, for gene enrichment with duplex DNA as well as with single-stranded DNA.

The isolation of a single copy gene and its flanking sequences in pure form from a typical eukaryotic genome requires enrichment by a factor of 10^5 to 10^6. None of the procedures described so far has been demonstrated to be effective for such a high degree of enrichment with reasonable yield for long DNA. Our goal is to develop such a method. In the present paper, we describe our initial tests on a much easier system: the *Drosophilia* ribosomal RNA (rRNA)[1] genes. In this case, because of the relatively small size of the genome and because the genes are approximately 185-fold reiterated (Tartof and Perry, 1970), the genes plus reiterated spacer sequences constitute about $1/_{150}$ of the genome.

The rRNA genes of *Xenopus laevis* differ sufficiently in base composition from the average for the entire genome so that enrichment by buoyant banding is effective (Birnstiel et al., 1968; Brown and Weber, 1968). In contrast to *Xenopus*, the G + C content of rRNA in *Drosophila* is only slightly higher (42.7%) than the G + C content (41%) of the total DNA (Ritossa et al., 1966; Hastings and Kirby, 1966). Thus, gene enrichment by DNA buoyant methods would probably be difficult. While our work was in progress, Wellauer and Dawid (1977) showed that it is possible to form R-loops with *Drosophila* rRNA and long duplex *Drosophila* rDNA and then purify the DNA with R-loops from the rest of the DNA by buoyant banding, based on the density shift due to the RNA. The density shift for an RNA:DNA hybrid is proportional to the ratio of the length of RNA:DNA hybrid to the length of the flanking DNA sequences; thus, this method is probably limited to long or tandemly repeated genes. It is our hope that the method described below will ultimately be applicable for gene enrichment even for short, nonreiterated genes.

The method is based on the affinity of the small molecule biotin to the protein avidin. Biotin reacts rapidly with avidin to form a very stable complex ($K_{diss} \simeq 10^{-15}$ M; Green, 1963). The overall procedures are summarized as follows.

(a) Biotin is covalently coupled to cytochrome *c* at a ratio of several biotins per cytochrome *c* by an *N*-hydroxysuccinimide ester acylation. The modified cytochrome *c* is then covalently attached to the RNA at a ratio of about 1 cytochrome to 130 nucleotides by formaldehyde cross-links, all as previously described (Manning et al., 1975a).

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[1] Abbreviations used are: rRNA, ribosomal ribonucleic acid; SSC, standard saline citrate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(b) This rRNA–cytochrome *c*–biotin is hybridized to long single strands of *Drosophila* DNA. The problem now is to separate the DNA:RNA–biotin hybrids from the rest of the DNA.

(c) One separation procedure is based on the covalent attachment of avidin to submicroscopic, water-soluble, poly(methyl methacrylate) spheres by a carbodiimide condensation. These polymer spheres, which are made by emulsion polymerization, have a diameter of 58 nm, an estimated molecular weight of about 7.6×10^7, and a buoyant density in CsCl of 1.27 g/cm^3 (Manning et al., 1975a). When DNA:RNA–biotin hybrids are incubated with avidin–spheres, some of the biotin sites on the hybrids bind spheres. The mixture is banded in CsCl. The spheres function as massive floats for those DNA strands (mol wt ca. 2×10^7) to which they are attached. These strands band at a low density relative to the bare DNA strands at $\rho = 1.71$ g/cm^3.

(d) An alternative to step c is conventional affinity chromatography. Avidin is attached to a solid support and the mixture of DNA:RNA–biotin plus unhybridized DNA is slowly passed through a column of this material. We have found that it is convenient and effective to attach the avidin to *N*-hydroxysuccinimide activated Corning controlled pore glass beads as the solid support.

(e) Removal of the DNA from either the DNA:RNA–avidin–sphere complex or the DNA:RNA–avidin–bead column is accomplished by denaturation of the hybrid with sodium hydroxide.

Our electron microscope studies of the arrangement of rRNA genes on the long single strands of rDNA enriched by the above procedure are described in a separate paper (Pellegrini et al., 1977).

Experimental Procedures

DNA and RNA. High molecular weight unlabeled *Drosophila melanogaster* DNA was extracted from a crude nuclear pellet prepared from organisms 16 to 24 h after puparium formation (Manning et al., 1975b).

^{3}H-Labeled *D. melanogaster* DNA was prepared from homogenized embryos cultured in the presence of [^{3}H]thymidine (C. Laird, personal communication). One gram of 0–8-h embryos was collected, washed in sterile H_2O, and dechorionated in 50% Clorox for 1 to 2 min. The embryos were thoroughly rinsed with sterile double distilled H_2O, dispersed in 5 mL of Schneider's medium containing 15% fetal calf serum, and homogenized using 8 strokes with a Type A Dounce homogenizer. The suspension was clarified by centrifugation at 100 rpm in a Sorval SS34 rotor for 1 min at 25 °C. The supernatant was removed and further centrifuged at 900 rpm for 7 min at 25 °C. The resultant pellet was gently resuspended in 1 mL of Schneider's medium–15% fetal calf serum containing 100 μL of [^{3}H]thymidine (67 Ci/mmol) and incubated at 25 °C for 24 h. The solution was adjusted to 0.5% sodium dodecyl sulfate–0.5 M $NaClO_4$–0.15 M NaCl–0.005 M Tris–0.05 M EDTA (pH 8.5) by adding appropriate volumes of 20% sodium dodecyl sulfate–5.0 M $NaClO_4$ and 0.45 M NaCl–0.015 M Tris–0.15 M EDTA (pH 8.5). DNA was then extracted as previously described (Manning et al., 1975b). The specific activity of a typical preparation of ^{3}H-labeled DNA was 2.5×10^5 cpm/μg under our standard counting conditions. ^{3}H-Labeled *E. coli* and pSC101 DNA were generous gifts from Dr. Mark Guyer.

The preparation of covalently closed pDm 103 plasmid DNA from *E. coli* K12 strain HB101 (pDm 103) was according to Glover et al. (1975). Closed circular pDm 103 DNA was converted to open circular DNA by exposure to X radiation (Sharp et al., 1970).

^{3}H-Labeled *E. coli* DNA coupled with cytochrome *c*–biotin, was prepared as described previously (Manning et al., 1975a) with the following modification: prior to dialysis against 0.01 M triethanolamine (pH 7.8), the DNA was sheared in 0.12 M sodium phosphate (pH 6.8) in a Virtis 60 blender at 14 krpm for 30 min at 4 °C. After shearing the single-strand weight average length of the DNA was measured as 2.2 kb by zone sedimentation through an alkaline 5–20% sucrose gradient.

The preparation of the *Drosophila* rRNA and its modification with cytochrome *c*–biotin have been described previously (Manning et al., 1975a).

Covalent Coupling of Avidin to a Solid Support. The solid support material used is the *N*-hydroxysuccinimidyl ester of glycophase controlled pore glass beads (Pierce Chemical Co.). One gram of the support material was added to 15 mL of 0.1 M $NaHCO_3$ containing 20 mg of avidin (Sigma Chemical Co.) at 4 °C. The mixture was degassed in vacuo, gently agitated for 12 to 16 h at room temperature, and stored at 4 °C. The number of biotin binding sites per cubic centimeter of avidin–glass beads was assayed by the addition of [^{14}C]biotin, followed by repeated washes with 1.0 M NaCl–0.005 M Tris–0.001 M EDTA (pH 8.5). One cubic centimeter of avidin–glass beads bound ca. 3×10^{-2} μmol of biotin.

Hybridization of E. coli DNA. The effect of the cytochrome *c*–biotin modification on the reassociation of a polynucleotide was studied with *E. coli* DNA. Nonradioactive *E. coli* DNA in 0.1 × SSC was denatured by the addition of 0.2 vol of 1.0 N NaOH. After 20 min at 25 °C the DNA was neutralized by the addition of 0.4 vol of 1.0 M Hepes (protonated form, Calbiochem). ^{3}H-Labeled DNA–cytochrome *c*–biotin in 1.0 M NaCl–0.001 M Tris–0.0002 M EDTA (pH 8.5) was added to a final [^{3}H]DNA to nonradioactive DNA ratio of 1:200. NaCl (5.0 M)–Tris (0.005 M)–EDTA (0.001 M) (pH 8.5) and twice recrystallized formamide were added to give a final concentration of 0.7 M Na^+ and 55% formamide. This solution was incubated at 35 °C for 0.5 to 1.5 h.

The extent of DNA reassociation was monitored by removing aliquots of the DNA sample at appropriate C_0t values. The aliquots were dialyzed against 0.12 M sodium phosphate (pH 6.8) at 4 °C for 24 h. The final volume of the aliquot was adjusted to 2.0 mL and the solution was fractionated on hydroxylapatite as described previously (Manning et al., 1975b). Recovery of DNA after dialysis was generally greater than 88%.

Hybridization of rRNA–Cytochrome c–Biotin to D. melanogaster DNA. [^{3}H]DNA (either relaxed plasmid DNA or total nuclear DNA) in 0.1 × SSC was denatured by the addition of 0.2 vol of 1.0 N NaOH. After 30 min at 25 °C the DNA was neutralized by the addition of 0.4 vol of 1.0 M Hepes. Ribosomal RNA–cytochrome *c*–biotin in 1.0 M NaCl–0.001 M Tris–0.002 M EDTA (pH 8.5) was added to a final DNA:RNA ratio of either 15:1 or 5:1. NaCl (5.0 M)–Tris(0.005 M)–EDTA (0.001 M) (pH 8.5) and twice recrystallized formamide were added to give a final concentration of 0.7 M Na^+ and 55% formamide. The solution was incubated at 35 °C for 0.5–1.5 h.

The reassociated sample was dialyzed against 1.0 M NaCl–0.005 M Tris–0.001 M EDTA (pH 8.5) at 4 °C with rocking for 5 h. The sample was then concentrated and fractionated on a 2 cm × 30 cm column of Sepharose 2B in 1.0 M NaCl–0.005 M Tris–0.001 M EDTA (pH 8.5). The elution of the DNA was monitored by radioactivity. This step removes

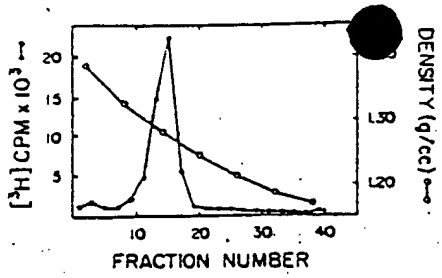

FIGURE 1: Buoyant banding experiment in CsCl of avidin-spheres labeled with [^{14}C]biotin.

much of the unhybridized rRNA. The peak fractions were pooled and further fractionated by use of avidin-spheres or avidin-glass beads.

Separation of rDNA:RNA Hybrid Molecules by Avidin-Spheres. The preparation and properties of the covalent conjugates of avidin to poly(methyl methacrylate) spheres have been described (Manning et al., 1975a). After hybridization to rRNA-biotin and fractionation on Sepharose 2B, the pooled DNA fractions were concentrated by vacuum evaporation to a volume of ca. 1.0 mL. Avidin-spheres (200 μL) (15 mg/mL in 1.0 M NaCl-0.005 M Tris-0.001 M EDTA (pH 8.5)) were added and the mixture was incubated at 4 °C for 16–20 h. Tris (0.05 M)–EDTA (0.005 M) (pH 8.5) was added to a final volume of 4.0 mL followed by CsCl to a final density of 1.284 g/cm^3. The mixture was centrifuged at 35 krpm for 24 h at 15 °C in a Spinco SW 50.1 rotor. The avidin-spheres formed a sharp band about ⅓ of the distance from the top of the tube at a density of 1.27 g/cm^3. The sphere band plus the region slightly above and below was gently removed with a broken tip Pasteur pipet. The remainder of the gradient was removed and the DNA pellet at the bottom of the tube was resuspended in 1.0 mL of SSC. Quantitation of the amount of DNA in the sphere band, the remaining gradient, and the pellet was based on the radioactivity present in each fraction.

DNA was separated from spheres by treatment with 1 M NaOH for 30 min at 25 °C. The solution was neutralized with 2 M Tris-HCl and the spheres pelleted by centrifugation for 30 min at 15 krpm in the Sorvall SS34 rotor.

Separation of rDNA:rRNA Hybrid Molecules by Avidin-Glass Bead Affinity Chromatography. rDNA:rRNA hybrid molecules formed with either total *Drosophila* nuclear [^{3}H]DNA or ^{3}H-labeled pDm 103 DNA were purified from nonhybrid molecules by passage of the pooled DNA fractions from the Sepharose 2B column over a 1-mL avidin-glass bead column in 1.0 M NaCl-0.005 M Tris-0.001 M EDTA (pH 8.5). The flow rate was regulated to 0.5 mL/min. The column was then washed with three 2.0-mL washes of 1.0 M NaCl-0.005 M Tris-0.001 M EDTA (pH 8.5) to elute the nonbound DNA fragments. DNA fragments containing a hybrid region were eluted by dissociating the hybrid region with either three 1.0-mL washes of 99% formamide or two 2.0-mL washes of 1.0 N NaOH. Quantitation of the amount of DNA in the 1.0 M NaCl-0.005 M Tris-0.001 M EDTA (pH 8.5) fractions, the formamide, and the NaOH fractions was based on the radioactivity present. Prior to hybridization with [^{125}I]rRNA, the fractionated DNA was gently deproteinized with an equal volume of water-saturated phenol.

Preparation of [^{125}I]rRNA. ^{125}I-Labeled rRNA was used to assay for the amount of rDNA in the several fractions. Radioactively labeled [^{125}I]rRNA was prepared essentially according to Orosz and Wetmur (1974). rRNA (an equimolar mixture of 18S + 28S) was dialyzed extensively vs. 0.01 M NaOAc (pH 5.0). Typically, a 30-μL reaction contained 2 μg of rRNA, 1 mCi of Na^{125}I (carrier free, Amersham Searle), and 2 × 10^{-4} M $TlCl_3$ in 0.1 M NaOAc (pH 5.0). The mixture was incubated for 30 min at 60 °C in a sealed glass tube and treated as described by Orosz and Wetmur (1974). The [^{125}I]rRNA was further purified by buoyant density banding in a sodium iothalamate (Mallinckrodt Pharmaceuticals) gradient (Serwer, 1975). Iothalamate was removed by extensive dialysis against 1.0 M NaCl-0.01 M Tris-0.001 M EDTA (pH 8.5). The RNA was stored in 2 × SSC at −20 °C. Specific activities of RNA preparations ranged from 5 × 10^6 to 8 × 10^7 cpm per μg.

Filter Hybridization of [^{125}I]rRNA to DNA. Quantitation of rRNA coding sequences in both fractionated and unfractionated DNA was achieved by filter hybridization with [^{125}I]rRNA. Membrane filter hybridization of [^{125}I]rRNA to DNA was carried out by the mini-filter technique exactly according to Kourilsky et al. (1974). Nitrocellulose filters (13 mm) containing 0.01 to 1 μg of denatured DNA were prepared. To these a 10-μL solution of [^{125}I]rRNA (0.8 μg/mL) in 2 × SSC was added and the filters were immediately immersed in mineral oil. They were incubated at 65 °C for 18 h and then treated with RNase as described by Kourilsky et al. (1974).

Solution Hybridization of [^{125}I]rRNA to pDm 103. The rate and extent of hybridization of [^{125}I]rRNA to the rDNA of pDm 103 were determined as follows: the hybridization solution contained 0.7 M NaCl, 0.001 M Tris, 0.0002 M EDTA (pH 8.5), 55% formamide, 0.025 μg/mL DNA, and 0.13 μg/mL [^{125}I]rRNA. Aliquots (100 μL) were sealed in small glass tubes and incubated at 35 °C to the appropriate R_0t values. The solution was then diluted to 1 mL with SSC and RNase A was added to a final concentration of 50 μg/mL. The mixture was at room temperature for 30 min. Carrier DNA (75 μg) was added and the nucleic acid molecules were precipitated by addition of 1.0 mL of cold 10% trichloroacetic acid and the precipitate was collected on glass fiber filters. The extent of hybridization of [^{125}I]rRNA to the plasmid was determined as RNase resistant counts.

Results and Discussion

CsCl Equilibrium Density Centrifugation of Avidin-Spheres. Figure 1 shows the results of the centrifugation of a solution containing 1 mg (1.8 × 10^{-5} μmol) of avidin-labeled poly(methyl methacrylate) spheres (avidin-spheres) and 1 μmol of [^{14}C]biotin (45 Ci/mol) in 5.0 mL of CsCl solution of average density 1.313 g/cm^3 for 48 h at 35 krpm in a Spinco SW 50.1 rotor. Fractions were collected by puncturing the bottom of the centrifuge tube. The density profile of the CsCl solution was determined from the refractive index of selected samples. The buoyant position of the avidin-spheres, as determined by the radioactivity present in the fractions, corresponded to a density of 1.27 g/cm^3.

The buoyant band of avidin-spheres in a CsCl density gradient can be identified readily because of its turbidity (Figure 2). This identification facilitates the removal of high molecular weight DNA:avidin-sphere complexes with minimum shear to the DNA.

The observation that avidin-spheres will specifically attach to DNA molecules which contain covalently bound cytochrome *c*-biotin has been previously reported (Manning et al., 1975a). The density at which the DNA:avidin-sphere complex bands in a CsCl equilibrium density gradient was determined as follows. A solution containing 1 mg of avidin-spheres and 0.35 μg of ^{3}H-labeled *E. coli* DNA (4 × 10^4 cpm per μg, 3.5 kb fragment length) with an average of one covalently bound cytochrome *c*-biotin per 130 nucleotides was incubated for 16

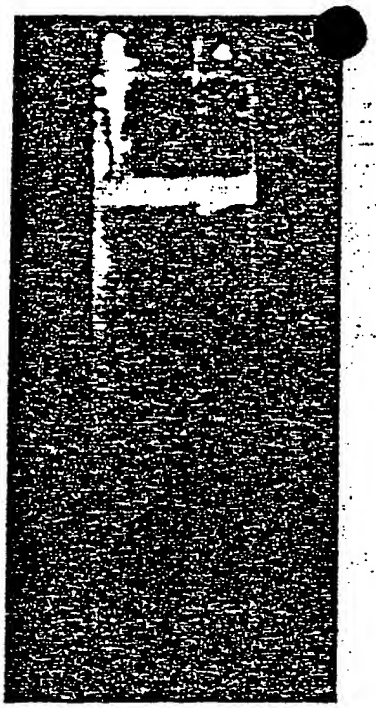

FIGURE 2: Photograph of a buoyant band of avidin spheres in a CsCl density gradient.

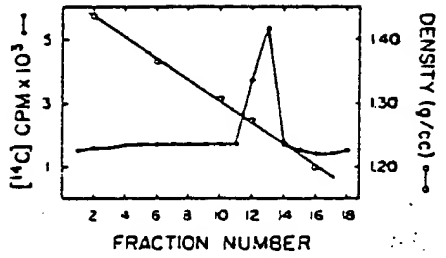

FIGURE 3: Banding of ^{3}H-labeled *E. coli* DNA covalently linked to cytochrome *c*-biotin and mixed with avidin-spheres in a CsCl density gradient; 100 μL of each fraction was spotted on a GFA filter and counted in toluene. The uniformly high background across the gradient represents Cl_3CCOOH soluble material.

h at 25 °C. Solid CsCl was added to the DNA-sphere solution to give an average density of 1.260 g/cm^3 and centrifuged for 48 h at 35 krpm at 25 °C in a Spinco SW 50.1 rotor. The gradient was fractionated into 10-drop fractions by puncturing the bottom of the tube. The position of the sphere band was determined by turbidity and the position of the DNA by radioactivity. As shown in Figure 3, the position of both the sphere band and the DNA band corresponded to a density of 1.27 g/cm^3. The DNA band at ρ = 1.27 g/cm^3 represented 70% of the total Cl_3CCOOH precipitable input counts, while the remainder of the DNA pelleted. Total recovery of the DNA by this procedure was 97%. Examination of the DNA found at ρ = 1.27 g/cm^3 in the electron microscope showed that 90% of all DNA fragments contained one or more attached avidin-spheres.

We believe that the DNA fragments which were removed with the sphere band but did not contain an attached sphere by electron microscopic observation are not due to nonspecific weak association of DNA with avidin-spheres but rather to breakage of large DNA molecules (one part of which initially contained an attached sphere) during gradient fractionation or spreading for electron microscopy.

When a CsCl density gradient experiment was performed with ^{3}H-labeled *E. coli* DNA with no attached cytochrome

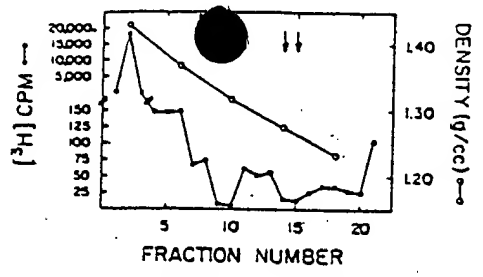

FIGURE 4: A mixture of avidin spheres and ^{3}H-labeled *E. coli* DNA which is not modified with cytochrome *c*-biotin is banded in CsCl. The position of the sphere band is indicated by arrows. Note the change of scale for the amount of [^{3}H]DNA.

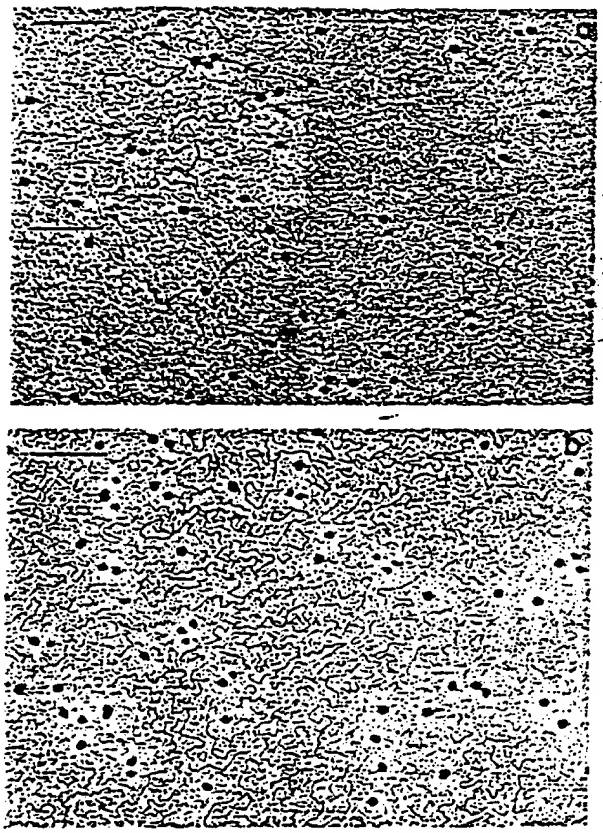

FIGURE 5: Electron micrographs of single-stranded *E. coli* DNA coupled to cytochrome *c*-biotin mixed with avidin-spheres and spread by the formamide-cytochrome *c* method (a) and of unmodified DNA spread with avidin-spheres (b). In a, arrows identify spheres attached to DNA. The calibration marker is 1 kb in length.

c-biotin and avidin-spheres, less than 0.05% of the DNA was present in the avidin-sphere band (Figure 4). Thus, there is only a very low level of nonspecific binding of unmodified DNA to the spheres.

This low level of nonspecific binding is shown in an electron micrograph (Figure 5) of unmodified and cytochrome *c*-biotin modified DNA spread with excess spheres in a formamide-cytochrome *c* spread. In the former case, no spheres are attached to DNA; in the latter case, as shown by the arrow, some spheres are attached to the DNA.

In conclusion, the CsCl density gradient and the electron microscope experiments show that the avidin-spheres, CsCl density gradient procedure is effective for separating DNA strands coupled to cytochrome *c*-biotin from those which are not.

Hybridization of Cytochrome-Biotin Labeled DNA with Unlabeled DNA. The gene enrichment procedure requires that nucleic acid molecules containing attached cytochrome *c*-

TABLE I: Gene Enrichment Tests on pD[redacted]3.[a]

DNA	Method of Enrichment	% of rRNA Coding Sequences in "Enriched" Fraction	% of rRNA Coding Sequences in "Depleted" Fraction	% of Total DNA in "Enriched" Fraction	% of Total DNA in "Depleted" Fraction
pSC 101	Avidin-sphere, CsCl			<0.01	>99.99
pDm 103 theor.		100	0	50	50
pDm 103	Avidin-sphere, CsCl	66	34	42	58
pDm 103	Avidin-sphere, CsCl	69	31	36	64
pSC 101	Avidin-glass bead column			<0.01	>99.99
pDm 103	Avidin-glass bead column	90	10	44	56

[a] Open circular [^{3}H]plasmid DNA was denatured and hybridized to rRNA–cytochrome *c*-biotin (130 nucleotides/cytochrome *c*-biotin). Hybridizations were carried out in 0.5-mL volumes in 55% formamide–0.7 M Na^+ at 35 °C with rRNA–biotin and plasmid DNA at concentrations of 30 and 2 μg/mL, respectively. The percent of rRNA coding sequences in each fraction was determined by membrane filter hybridization with [^{125}I]rRNA as described under Experimental Procedures. The percentage of total DNA in the "enriched" or "depleted" fraction was determined as Cl_3CCOOH precipitable counts and normalized to 100%. Recovery of total Cl_3CCOOH precipitable input counts was greater than 97%.

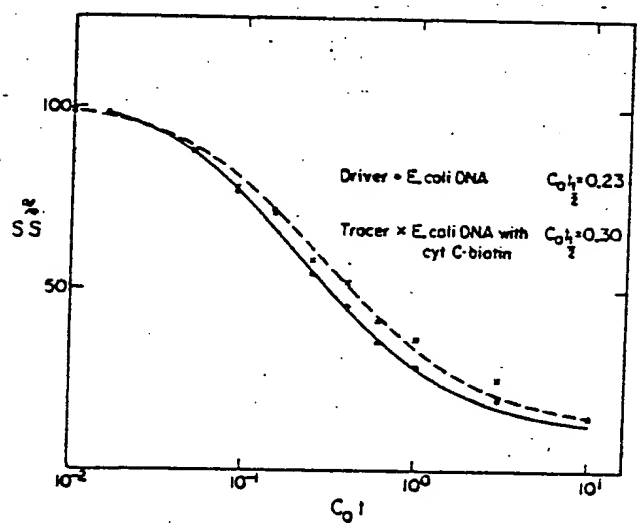

FIGURE 6: Reassociation rate, as measured by hydroxylapatite chromatography of excess unmodified unlabeled DNA with itself and of cytochrome *c*-biotin modified [^{3}H]DNA with the unmodified driver DNA.

biotin be capable of hybridizing with unlabeled nucleic acid molecules. We have compared the hybridization rate of cytochrome *c*-biotin labeled DNA with unlabeled DNA to the reassociation rate for unlabeled DNA. Cytochrome *c*-biotin was attached to single-stranded ^{3}H-labeled *E. coli* DNA of average fragment length 2.2 kb to give a final ratio of 130 nucleotides per attached cytochrome *c*-biotin. Nonradioactive *E. coli* DNA of average fragment length 2.2 kb was denatured and added in 200-fold excess to the cytochrome *c*-biotin–[^{3}H]DNA. Samples were reassociated to a variable set of C_0t values and fractionated by hydroxylapatite chromatography.

As shown in Figure 6, the reaction rate of the modified DNA with unmodified DNA is only 0.23/0.30 slower than the reassociation rate of unmodified strands. We do not know whether the small difference in rate is real or is experimental error, but the main point is that the rates are almost the same. The effect of the modification reaction on the thermal stability of the hybrids was not measured. We can only say that the hybrids remain duplex at 60 °C in 0.12 M sodium phosphate (pH 6.8), which are the hydroxylapatite fractionation conditions used.

Gene Enrichment Tests on pDm 103. The recombinant plasmid pDm 103 consists of the procaryotic vector, pSC 101, of length 9.2 kb and a *Drosophila* fragment of length 17.1 kb which contains one copy of the 18S and 28S rRNA coding sequences (Glover et al., 1975).

The lengths of the 18S and 28S rRNA genes of *Drosophila* have been measured as 2.2 and 4.3 kb, respectively (Pellegrini et al., 1977; Wellauer and Dawid, 1977; White and Hogness, 1977). Thus, the coding sequences constitute 12.3% (6.5/52.6) of the plasmid.

The rate and extent of hybridization of an excess of ^{125}I-labeled 18S and 28S rRNA with pDm 103 were measured as described under Experimental Procedures. The saturation hybridization value was 11.5 ± 2.5% of the total plasmid DNA, in agreement with the calculation above. The observed $R_0t_{\frac{1}{2}}$ value was 5.8×10^{-3} mol s L^{-1} in a 0.7 M Na^+–55% formamide solvent at 35 °C. This value is approximately as expected for an RNA driven RNA:DNA reaction rate for an RNA of this complexity (J. W. Casey, personal communication).

Since only one of the strands of pDm 103 contains the coding sequences for the rRNAs (Glover et al., 1975), this DNA is an excellent test system for the proposed gene isolation procedures. The results of such tests, which are presented in Table I, show the following. (a) The binding of the control DNA (pSC 101) by avidin-spheres and by avidin-glass beads is less than 0.01% in both cases. (b) Of the rRNA genes 65 to 70% are selected in the "enriched" fraction by the avidin-sphere procedure; 30 to 35% are not. If all of the DNA strands were full length, 32 to 35% of the total DNA should then be in the "enriched" fraction, in approximate agreement with observation. The slightly higher value (42%) observed in one of the two experiments may be due to some DNA reassociation. (c) Ninety percent of the genes and 44% of the DNA are selected by the avidin–glass bead affinity column, thus indicating efficient isolation of the genes with little strand breakage.

TABLE II: Gene Enrichment Experiments on Total *Drosophila* DNA.[a]

DNA	Method of Enrichment	% of rRNA Coding Sequences in "Enriched" Fraction	% of rRNA Coding Sequences in "Depleted" Fraction	% of Total DNA in "Enriched" Fraction	% of Total DNA in "Depleted" Fraction	Enrichment Factor (% of Total rRNA + Spacers)
Total nuclear DNA, theor.		100	0	0.64	99.36	156 (100)
Total nuclear DNA	Avidin–sphere, CsCl[b]	20	80	0.30	99.70	66 (42)
Total nuclear DNA	Avidin–sphere, CsCl[b]	25	75	0.20	99.80	124 (80)
Total nuclear DNA	Avidin–glass bead column[c]	57	43	0.77	99.23	74 (47)

[a] The calculation that rDNAs (genes plus spacers on the coding strand) make up $1/156$ of the total *Drosophila* DNA is based on the following. The average total length of the spacer sequences in *Drosophila* rDNA has been measured as 7.25 kb (Pellegrini et al., 1977). About 37% of the 28S sequences contain an insertion of average length 4.57 kb. Thus, the average total length of a rDNA repeat unit is 15.46 kb. The average length of 18S plus 28S gene sequences is 6.5 kb. The gene sequences make up 0.27% of the genome. The total rDNA, therefore, makes up 0.0027 $(15.46/6.5) = 1/156$ of the genome. [b] $R_0t = 0.016$. [c] $R_0t = 0.13$. Hybridizations were performed at RNA–biotin and DNA concentrations of either 6 and 90 μg/mL, respectively, or 7.5 and 35 μL, respectively. The percent of rRNA coding sequences in each DNA fraction was determined by membrane filter hybridization with [^{125}I]rRNA.

Isolation of rRNA Coding Sequences from D. melanogaster Nuclear DNA. The experiments for the isolation of DNA fragments which contain the rRNA coding sequences from total *D. melanogaster* nuclear DNA are identical, in principle, with those described for the isolation of the rRNA coding strand in pDm 103 DNA. They differ, however, in the initial amount of DNA required. Since in *Drosophila* only 0.27% of the nuclear DNA is rRNA coding sequences, the amount of DNA required for an accurate measurement of the degree of enrichment is between 1 and 2 mg per experiment. Using this amount of starting material, the DNA is denatured and hybridized with an excess of cytochrome *c*–biotin coupled rRNA. The ratio of RNA to DNA in the hybridization mixture was either 1:5 or 1:15. This ratio represents a 74- to 24-fold excess of rRNA over rDNA. Following hybridization to saturation ($R_0t = 0.13$ or 0.016 mol s L^{-1}, which is approximately 400 or 30 times the observed $R_0t_{1/2}$ of 5.3×10^{-3} mol s L^{-1}), the RNA:DNA hybrid molecules were selectively removed by either binding the hybrids to avidin–spheres and banding the complex in a CsCl equilibrium gradient or by binding the hybrids to an avidin–glass bead column. The results of the enrichment of DNA strands containing rRNA coding sequences by the above selection procedures are shown in Table II.

The 185-fold reiterated 18S and 28S rRNA genes make up 0.27% of the *Drosophila* genome (Tartof and Perry, 1970). The several spacer sequences between the tandemly repeated genes are, on the average, slightly greater in length than the genes for the mature rRNAs, so that the total amount of gene plus spacer on the coding strand is 0.64% or $1/156$ of the total DNA. The details of this calculation are given in the legend to Table II.

An enrichment factor of 156 would mean that the rDNA was 100% pure. In the several experiments reported in Table II, enrichment factors of 66 to 124 were achieved, giving long rDNA strands which are 42 to 80% pure.

In the avidin–sphere enrichment experiments, 20 and 25% of the total rDNA was recovered in the enriched fraction. In the avidin–glass bead experiment, 57% was recovered.

Electron microscope observations showed that the single-strand length of the DNA starting material was primarily in the range of 70 to 100 kb. The lengths of the molecules of enriched DNA recovered from the avidin–sphere experiments were mostly in the range of 50 to 70 kb for the avidin–glass bead experiments they were 20 to 30 kb. Thus, in this respect, the sphere banding procedure is better. It may be, however, that with further attention to conditions for very gentle passage of the DNA over the solid support, it will be possible to avoid breakage of the strands in this procedure.

Further Discussion

The experiments described here demonstrate that rRNA:rDNA hybrid structures can be selectively isolated from total nuclear DNA in sufficient quantity, of sufficient purity, and of sufficient single-strand length for an electron microscope study of the sequence organization of the genes (Pellegrini et al., 1977). Although this approach has been successful in the isolation of repetitive genes, we believe that further tests will be necessary to determine its usefulness in the isolation of single copy genes and their flanking sequences.

Acknowledgments

We are grateful to our colleagues L. Angerer, T. Broker, N. D. Hershey, and P. Yen who have generally contributed to the development of avidin–biotin methodology, to N. D. Hersey who developed the method of preparing spheres, and to M. Grinder for the preparation of nucleic acids.

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An Endonuclease from Calf Liver Specific for Apurinic Sites in DNA†

J. Philip Kuebler and David A. Goldthwait*

ABSTRACT: An endonuclease specific for apurinic sites in double-stranded DNA has been partially purified from calf liver extracts. The enzyme has a pH optimum of 9.5, is only slightly stimulated by low concentrations of Mg^{2+}, and has a molecular weight of 28 000. Inhibitors of the endonuclease include Ca^{2+}, EDTA, *p*-HOHgBzO, NaCl, and tRNA. The enzyme introduces single- and double-stranded breaks in depurinated DNA. High concentrations of the enzyme preparation degrade untreated single-stranded DNA, but not ultraviolet (UV) irradiated DNA or DNA treated with methylmethanesulfonate or 7-bromomethyl-12-methylbenz[*a*]anthracene. Enzymatic incisions produce 3′-hydroxyl and 5′-phosphate end groups. Some of the properties of the calf liver apurinic endonuclease differ from those of a similar endonuclease obtained from calf thymus by S. Ljungquist and T. Lindahl [(1974), *J. Biol. Chem. 249*, 1530] and in this laboratory. The data suggest that these are isozymes.

Deoxyribonucleic acid in cells may undergo depurination damage by various pathways. Base release by either chemical or enzymatic reactions can occur after the bases are altered by specific alkylating agents or γ irradiation. Lindahl and Nyberg (1972) demonstrated the slow spontaneous hydrolysis of purines under physiological conditions and calculated that up to 3% of the total purines could be lost during the lifetime of cells such as human neurones if there was no replacement. An endonuclease active only at depurinated sites was isolated from *Escherichia coli* by Paquette et al. (1972), while a preparation active on depurinated sites and alkylated DNA (Hadi and Goldthwait, 1971) has now been resolved into an enzyme active on depurinated sites and one active on alkylated DNA (endonuclease II, Kirtikar et al., 1976a). Evidence for the existence in mammalian tissues of endonucleases capable of recognizing depurinated sites was obtained by Verly and Paquette (1973). Ljungquist and Lindahl (1974) purified an endonuclease 830-fold from calf thymus and reported many of its properties. Subsequently, an enzyme active on apurinic sites in DNA was demonstrated in a number of human cell lines (Teebor and Duker, 1975) and an altered specific endonuclease for depurinated sites has been found in extracts of two cell lines from xeroderma pigmentosum patients (Kuhnlein et al., 1976). This paper reports the isolation and partial purification from calf liver of an endonuclease active on depurinated DNA which possesses some properties that differ from those observed with the enzyme obtained from calf thymus. The possibility of apurinic endonuclease isozymes is discussed.

Materials and Methods

Reagents. [^{3}H]Thymidine was obtained from the New England Nuclear Corp., Boston, Mass. Sodium borohydride (98% pure) was purchased from the Fisher Scientific Co., Fairlawn, N.J. Dithiothreitol and 2-mercaptoethanol were both from Sigma Chemical Co., St. Louis, Mo. Toluenesulfonyl fluoride, 97%, was obtained from Aldrich Chemical Corp., Milwaukee, Wis. 1,3-Bis[tris(hydroxymethyl)methylamino]propane (BTP buffer),[1] A grade (pK_{a_1} = 9.0, pK_{a_2} = 6.8), was purchased from Calbiochem, Los Angeles, Calif. DNase I was also obtained from this source. Bovine spleen and snake venom phosphodiesterases were obtained from the Worthington Co.

DNA. The procedure for preparing [^{3}H]thymine-labeled *Bacillus subtilis* or *Escherichia coli* DNA of Smith (1967) was employed. Specific activity of the DNA was approximately

† From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106. *Received October 13, 1976.* This work was supported by a grant from the National Institutes of Health (CA 11322) and a contract with ERDA (11-1) 2725. D.A.G. was supported by National Institutes of Health Research Career Award No. KO-6-GM-21444.

[1] Abbreviations used are: BTP buffer, 1,3-bis[tris(hydroxymethyl)-methylamino]propane-hydrochloric acid buffer; EDTA, ethylenediaminetetraacetic acid, sodium salt; *p*-HOHgBzO, *p*-hydroxymercuribenzoate; AP, alkaline phosphatase.

Chromosoma (Berl.) 53, 107—117 (1975)

A New Method of *in situ* Hybridization*

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Abstract. A new method for gene mapping at the chromosome level using *in situ* hybridization and scanning electron microscopy is described and has been applied to mapping the rRNA genes of *Drosophila melanogaster*. Biotin is covalently attached to *Drosophila* rRNA *via* a cytochrome c bridge at a ratio of one cytochrome-biotin per 130 nucleotides by a chemical procedure. Polymethacrylate spheres with a diameter of ca. 60 nm are prepared by emulsion polymerization and are covalently attached to the protein avidin at a ratio of 5–20 avidins per sphere. The biotin-labeled rRNA is hybridized to denatured DNA in a chromosome squash. Upon incubation with a sphere solution, some of the biotin sites become labeled with spheres because of the strong non-covalent interaction between biotin and avidin. The chromosome squash is examined in the scanning electron microscope (SEM). Polymer spheres, which are visible in the SEM, are observed to label the nucleolus, where the rRNA genes are located.

Introduction

The *in situ* hybridization technique was developed to map sites of specific genes and other interesting genetic sequences on chromosomes (Gall and Pardue, 1969; Pardue and Gall, 1969). The technique depends on the preparation of radio-labeled RNA or DNA probes complementary to sequences in the chromosome. These probes, which must be of high specific activity, and usually contain either ^{3}H or ^{125}I as the radioactive isotope, are hybridized to denatured DNA in a chromosome squash. The position of the complementary sequence in the chromosome is determined by light microscope autoradiography.

We wish to describe our initial studies of an alternative method of *in situ* hybridization which uses scanning electron microscopy and a non-radioactive label attached to the polynucleotide chain. For these initial tests we have chosen the 16S and 26S rRNA genes localized in the nucleolus and/or at the base of the X chromosome in the polytene chromosomes of *Drosophila melanogaster*. Further studies and developments are necessary before the sensitivity and resolution of this new method can be evaluated and compared to those of autoradiographic methods.

The basic features of our method are as follows.

(1) The labels are poly(methylmethacrylate) spheres with a diameter of ca. 60 nm. They are prepared by emulsion polymerization, chemically modified, and then covalently coupled to the protein avidin, so that there is a ratio of a few avidin molecules per sphere (avidin-spheres).

* Contribution number 5121 from the Department of Chemistry.

(2) Biotin is covalently coupled to the RNA of interest (biotin-RNA) at a ratio of about one biotin per 100 to 200 nucleotides.

(3) Salivary gland chromosomes from isolated nuclei are spread on a glass slide and photographed in the light microscope (Mitchell and Lipps, 1975).

(4) The chromosomal DNA is denatured and hybridized with the biotin-RNA essentially as described by Pardue *et al.* (1970).

(5) The hybridized RNA-biotin is incubated with a suspension of avidin-spheres and prepared for visualization in the scanning electron microscope (SEM).

The labeling reaction is based on the fact that avidin and biotin combine rapidly to form a remarkably stable complex (Green, 1963). Heitzmann and Richards (1974) have already described the use of the biotin-avidin interaction in electron microscope labeling procedures. We wish also to note that the use of polymer spheres for labeling studies has been described by Molday *et al.* (1974, 1975). Our studies on the use of the biotin-avidin reaction and of polymer spheres for nucleic acid labeling problems were initiated independently.

Materials and Methods

1. Preparation of Radioactive rRNA. Ribosomal RNA containing a ^{3}H-uridine label was obtained by microinjection of mid-third instar larvae with a dose of approximately one millicurie of ^{3}H-uridine (70ci/mmole) per 250–300 larvae. After 4 hours incubation at 25°C the larvae were collected and washed with 0.1 × SSC (0.015 M NaCl, 0.0015 M Na citrate, pH 7.0). Polyribosomes were isolated and rRNA extracted by procedures described by Boshes (1970). The 26S and 16S rRNA components were further purified by velocity sedimentation through a 5 to 20% sucrose gradient in 0.1 M NaCl, 0.01 M acetate, pH 5.1, at 62 krpm for 2 hours at 4°C in the Spinco SW65 rotor. The 26S and 16S regions of the gradients were pooled and rRNA precipitated with two volumes of 80% ethanol. The rRNA was resuspended in 0.1 M NaCl, 0.01 M sodium acetate, pH 5.1, and stored at −20°C.

2. Preparation of rRNA-Cytochrome c-Biotin. A cytochrome c bridge was used to attach biotin to the rRNA probe. The N-hydroxysuccinimidyl ester of biotin (NHS-B) was used to acylate some of the amino groups of cytochrome c, following the procedures of Becker *et al.* (1971) and Heitzmann and Richards (1974).

NHS-B was prepared as follows: A mixture of 3 millimoles of unlabeled biotin and 80 µl of a 100 µc/ml solution of ^{14}C-biotin (50 µcurie/µmole, Amersham/Searle) was evaporated to dryness and dissolved in 9 ml of dimethylformamide at 60°C. Three millimoles of N-hydroxy-succinimide were added and dissolved followed by 4 millimoles of dicyclohexylcarbodiimide. This solution was stirred at room temperature overnight. A white precipitate of the dicyclo-hexylurea formed in about 5–10 minutes. The solution and precipitate were separated and the solution reduced to dryness by rotary evaporation. The resulting solid was washed with several volumes of hot methanol. The remaining solid contained the bulk of the ^{14}C counts (~7000 cpm/mg). This precipitate, although probably not pure, contained the NHS-B in a form suitable for its subsequent use.

A 2.0 µl solution containing 10 mg/ml of cytochrome c in 0.1 M $NaHCO_3$ was mixed with 0.2 ml of dimethylformamide containing 1.6 mg (~4.7 µmoles) of the radioactive NHS-B. The mixture was incubated at room temperature for 1 hour, passed over a 15 × 2 cm Sephadex G-25 column and eluted with 0.15 M NaCl. The cytochrome c fraction was collected and stored at 4°C. 100% of the input biotin was covalently attached to the protein (3 biotin per cyto-chrome c).

Biotin-labeled cytochrome c was coupled to rRNA by reaction with formaldehyde using a modification of the procedure of Brutlag *et al.* (1969). 0.05 ml of a cytochrome c-biotin solution (3 mg/ml) was added to 1.0 ml of an rRNA solution (100 µg/ml) after each had been dialyzed against 0.01 M triethanolamine (TEA), pH 7.8. 0.11 ml of 6% formaldehyde in the

ame buffer was added, and the mixture incubated at 37°C for 30 minutes. Formaldehyde was emoved by dialysis against four or more changes of TEA buffer at 4°C for 48 hours; 5.0 M NaCl was then added to give a final concentration of 1.0 M. This solution was fractionated on a 28×1.3 cm column of Sepharose 6B in 1.0 M NaCl, 0.001 M EDTA, pH 8.0. The elution of the RNA and of the cytochrome c was detected by absorbance at 260 nm and 410 nm, respectively. Extinction coefficients of 25 cm^2/mg for the RNA and 7.7 cm^2/mg for the cytochrome c were used to calculate a nucleotide to cytochrome ratio of 130:1 in the product. The biotin-rRNA was dialyzed against 2×SSC, pH 7.5. For storage, the sample was divided into small fractions and frozen at −70°C.

It should be noted that this procedure relies on the fact that the positively charged protein, cytochrome c (pI ≈ 10.5), binds by electrostatic interactions to RNA at low ionic strength (0.1 M) thus favoring the formaldehyde crosslinking reaction. Any cytochrome c which is not covalently bound to the nucleic acid is dissociated at the high ionic strength (1.0 M) used in the Sepharose column fractionation (Olivera, 1966).

3. Preparation and Properties of Polymer Spheres. Polymer spheres were prepared by emulsion polymerization. Methyl methacrylate (2.25 g, Polysciences), ethylene dimethacrylate (0.60 g, Polysciences), methacrylic acid (0.15 g, Polysciences), water (47 ml, redistilled) and sodium dodecyl sulfate (0.12 g, MC&B, USP grade) were measured into a 100 ml round bottom flask and mixed thoroughly. The mixture was sonicated for a few seconds (power 4, Branson Sonifier Model S-125) to produce a white emulsion. Argon was bubbled through the emulsion for several minutes. A solution of 60 mg of $K_2S_2O_8$ in 10 ml water was then added to the stirred emulsion, and deoxygenation with argon was repeated. The flask was stoppered with a rubber septum and heated at 60°C with stirring for 38 hours. The emulsion was stored at 4° in 1 mM EDTA.

The emulsion was treated with NaOH in order to hydrolyze some of the ester groups thus increasing the surface concentration of carboxylate anions and probably displacing some of the SDS from the sphere surface. Equal volumes of a sphere emulsion, as described above, and 2 M NaOH were mixed and heated at 85°C for 100 hours. The emulsion was dialyzed exhaustively at 4° against 0.10 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA (pH 7.0), then against water, and finally against 1 mM EDTA. The resulting sphere emulsion was stored at 4°C.

Sphere concentrations were estimated by weighing the residue left on evaporation of a weighed aliquot of emulsion which had been exhaustively dialyzed against water. In order to measure the diameter of the spheres and to observe their general morphology, they were mounted for electron microscopy from a sphere concentration of 40–50 μg/ml by the formamide modification of the Kleinschmidt cytochrome c procedure. The hyperphase was 50% formamide, 0.10 M Tris, 0.01 M EDTA (pH, 8.5) and the hypophase was 17% formamide, 0.01 M Tris, 0.001 M EDTA (pH 8.5). The film was rotary shadowed with Pt-Pd. A typical transmission electron micrograph (Fig. 1a) shows the spheres to be approximately round and well dispersed. The measured diameters gave a rather narrow symmetrical distribution curve with a peak at 58 nm and with 67% of the values falling within the range 45–62 nm. Assuming a density of 1.24 g/ml, the average molecular weight of a sphere with a diameter of 58 nm is calculated to be 7.6×10^7 daltons. Fig. 1b illustrates the appearance of the spheres as photographed in the scanning electron microscope at high magnification. A solution containing 0.5 μg of spheres in 5 ml of cesium chloride solution of average density 1.20 g/ml was centrifuged for 40 hours at 100000 g. The position of the sphere band was readily observed from the turbidity and corresponded to a density of 1.27 g/ml. Spheres banded in sodium iothalamate solution (Serwer, 1975) at a density of 1.226.

4. Covalent Coupling of Avidin to Spheres. Avidin-labeled polymethacrylate spheres (avidin-spheres) were prepared by coupling avidin (Sigma) and spheres with 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide methyl p-toluene sulfonate (Aldrich).

Spheres at a concentration of 21 mg/ml were suspended in 1.0 mM EDTA. Then 0.72 ml of 0.05 M carbodiimide solution was added to 3.6 ml of the sphere suspension, followed by 1.44 ml of avidin solution (2 mg/ml). The mixture was incubated at 25°C for 16 hours and the reaction was quenched by addition of 0.01 ml of neat 2-ethanolamine. 5.0 M NaCl was added to a final concentration of 1.0 M. The mixture was divided into 1.0 ml aliquots and layered onto a 3.0 ml, 5 to 20% sucrose gradient over a 1.0 ml cushion of 60% sucrose, 1.0 M

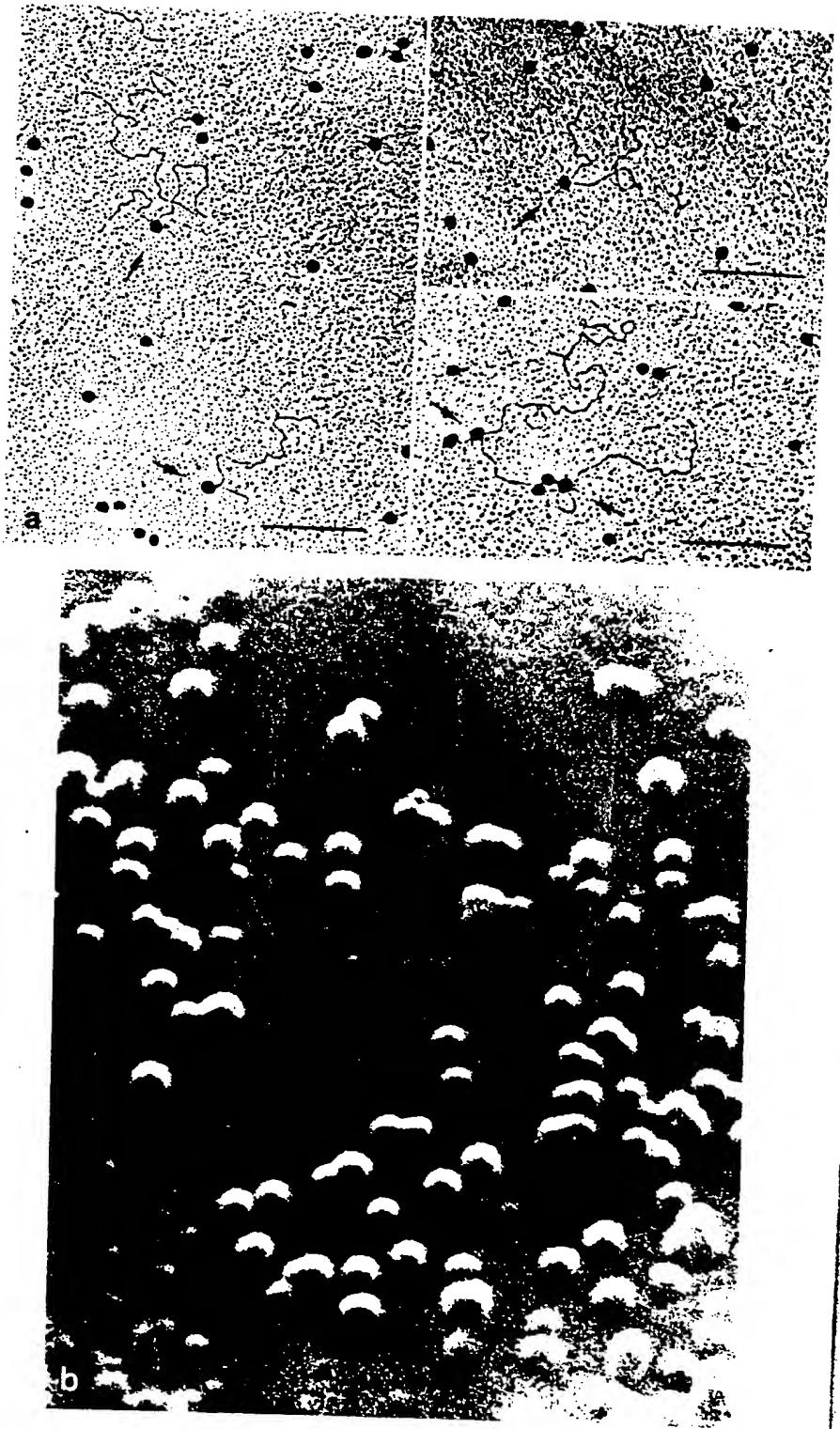

Fig. 1a and b

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NaCl, 0.01 M EDTA, pH 8.5. After centrifugation for 1 hour in the Spinco SW50.1 rotor at 35 krpm at 15°C, the avidin-spheres had migrated to the top of the sucrose cushion. The sphere band was removed by dripping and dialyzed for 16 hours at 25°C against 1.0 M NaCl, 0.01 M EDTA, pH 8.5. The spheres were again centrifuged as above and finally stored in 1.0 M NaCl, 0.01 M EDTA, pH 8.5 at 4°C.

Part of the rationale for this preparative and separation procedure is that the positively charged protein, avidin, is electrostatically bound to spheres at low electrolyte concentration but dissociated, unless covalently coupled, at high electrolyte concentration.

The number of biotin binding sites per sphere was assayed by testing the avidin spheres with ^{14}C-biotin, followed either by repeated dialysis to remove unbound biotin or by exclusion chromatography through Sepharose 6B. The number of biotin binding sites per avidin-sphere, prepared as described, was 19. Pure avidin, with a molecular weight of 66000, binds four biotins per molecule (Green and Toms, 1970). Our starting preparation bound about 3.2 millimoles of biotin per 66000 mg of protein. It is not known how many of the binding sites are inactivated when avidin is covalently linked to the spheres. Thus, the number of bound avidins may reasonably be estimated to lie between 5 and 19.

5. Chromosome Preparation and in situ Hybridization. Chromosome preparations from isolated nuclei were made essentially as described by Mitchell and Lipps (1975). Chromosomes on slides were first photographed under phase contrast. The cover slips were removed from the slides after freezing in liquid nitrogen. The slides were rinsed in 100% ethanol and air dried. The procedure for *in situ* hybridization is essentially as described by Pardue *et al.* (1970). The slides were rinsed in 2 × SSC, incubated in 0.07 M NaOH for 2 minutes to effect denaturation, washed twice in 70% ethanol and twice in 95% ethanol before drying. Hybridization was carried out at a concentration of rRNA-biotin of 2 μg/ml in 2 × SSC, 50% formamide for 16–20 hours at 25°C (Alonso, 1973). The slides were rinsed four times in 2 × SSC. 300 μl of a solution of avidin-spheres (40 μg/ml in 1.0 M NaCl, 1 mg/ml cytochrome c) was placed over the chromosomes. The slides were maintained at 25°C for 4 hours, extensively rinsed in 2 × SSC, 70% and 95% ethanol and then allowed to air dry. That portion of the slide containing the chromosome material was cut to a 2 cm square, mounted on an aluminum stud, and coated with carbon and/or gold for examination in the SEM.

Results

In *in situ* hybridization experiments Pardue *et al.* (1970) found the rDNA of *Drosophila hydei* solely within the nucleolus. Their results are consistent with biochemical and genetic studies on *D. melanogaster* that show the genes to be located in or near the nucleolus organizer region (Ritossa and Spiegelman, 1965), which maps at the base of the X chromosome. Therefore the region of particular interest in the present study is the nucleolus.

Chromosomes mounted directly for SEM by the methods of Mitchell and Lipps (1975) have very clear morphological features including a banding pattern which correlates well with the pattern seen by light microscopy. However, treatment with NaOH, which is necessary for *in situ* hybridization, causes flattening of the chromosomes and loss of many of the recognizable morphological features.

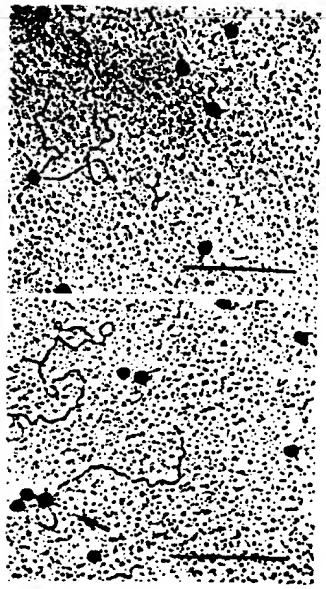

Fig. 1a and b. Transmission and scanning electron micrographs of polymer spheres. (a) Biotin was attached to calf-thymus DNA via a cytochrome c bridge by the procedures described in the text for rRNA, at a ratio of one cytochrome c-biotin per 700 nucleotides. This solution was incubated with avidin-spheres, and mounted for electron microscopy by the formamide modification of the standard Kleinschmidt procedure (Davis, Simon, and Davidson, 1970). The spheres are very opaque because the grids were rotary shadowed with Pt-Pd. The spheres have an average diameter of 58 nm; the calibration bar is 1,000 nucleotides or 0.32 μm. (b) High magnification SEM picture of spheres on a chromosome squash. ca ×100,000

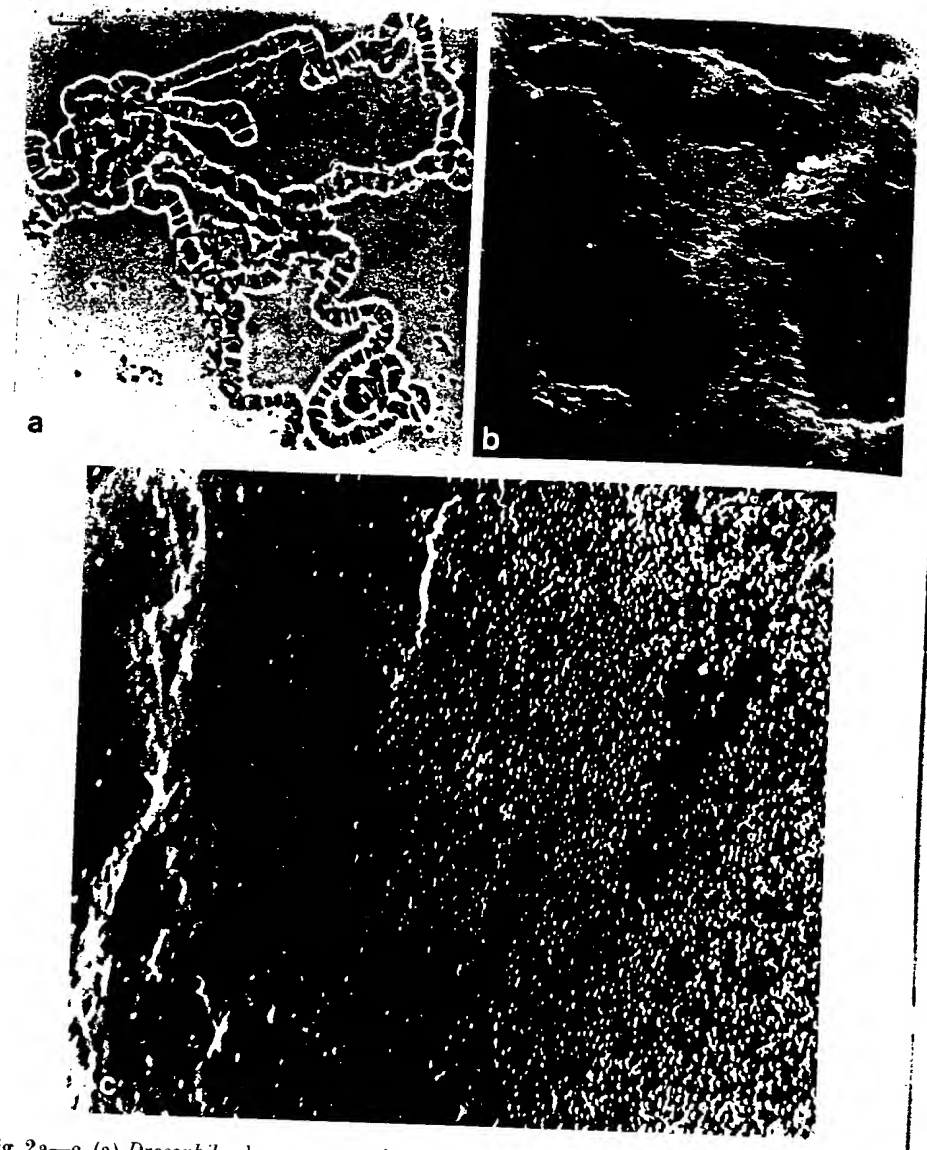

Fig. 2a—c. (a) *Drosophila* chromosomes photographed by phase optics in the light microscope *prior* to denaturation and hybridization. (b) Low magnification SEM picture of the region around the chromocenter and nucleolus after *in situ* hybridization. Bars in (a) and (b) represent 10 μm. (c) Higher magnification SEM picture of the region including part of the X chromosome and part of the nucleolus, as indicated in the square and parallelogram in the top pictures. The nucleolus is densely labeled with spheres. ca. ×10,000

The general silhouette of the chromosomes as viewed from above is, however, still clearly recognizable. Positions on the chromosomes can be identified by correlation of the SEM pattern with the photomicrograph obtained under phase optics prior to the NaOH treatment.

Fig. 3a and b. (a) Li
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tip of the X chromo
graph) after *in situ*

Fig. 2 shows t
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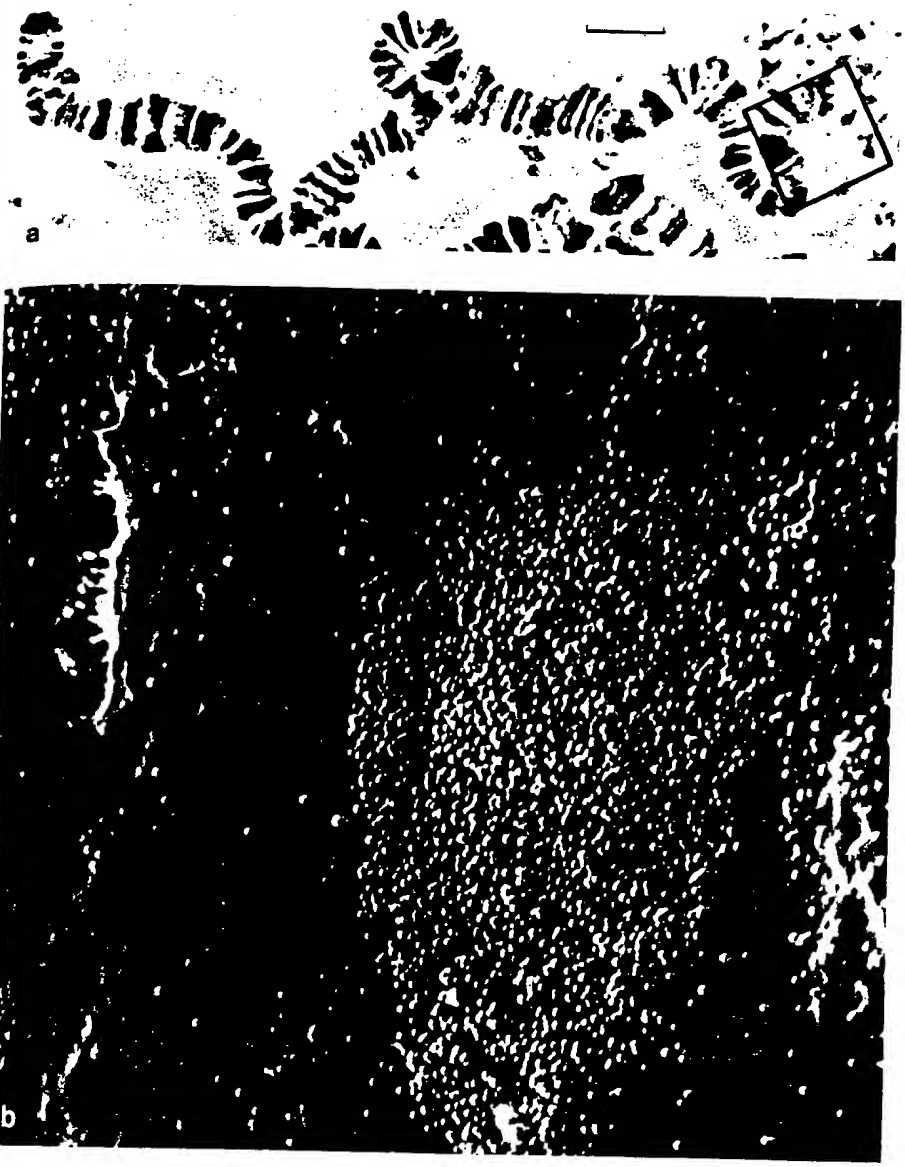

Fig. 3a and b. (a) Light micrograph of a detached X chromosome with a nucleolus at its base, prior to denaturation and hybridization. Bar represents 10 μm. (b) SEM picture of part of the tip of the X chromosome and the nucleolus (the region within the square in the light micrograph) after *in situ* hybridization, showing a dense labeling of the nucleolus with spheres. ca. ×15,000

Fig. 2 shows the results of a typical hybridization experiment. The light micrograph of the chromosomes (Fig. 2a) shows that the nucleolus occurs at the chromocenter and close to the base of the X chromosome. In a low magnification SEM picture (Fig. 2b) the general morphology of the chromosomes in this region

Fig. 4a and b. (a) An X chromosome fixed in 1:1 acetic:lactic acid, with no visible nucleolus. **Bar represents 10 μm.** (b) As shown in the SEM, after *in situ* hybridization, there is a low, but above background, density of spheres at the tip of the X chromosome. ca. ×15,000

is apparent. At this magnification the polymer spheres are visible as small dots. A high density of spheres covers the nucleolus, while a much lower sphere density over other regions of the chromosome and over the rest of the slide is apparent. The spheres may be seen more clearly in the moderately high magnification SEM picture of a small section of the nucleolus (Fig. 1c). There are very few spheres over the adjoining regions of the X chromosome. The chromosome arms and the contaminating cytoplasmic material were carefully examined; no other position with a significant number of spheres was observed.

Fig. 3 shows another typical result. In this case, the pressure applied during chromosome squashing has resulted in the detachment of the X chromosome from the chromocenter. A portion of the nucleolus remains attached to the base of the X chromosome (Fig. 3a). The high magnification SEM picture of this region (Fig. 3b), after hybridization and labeling, shows a very high density of spheres over the nucleolus.

In chromosome preparations treated with 45% acetic acid (such as shown in Figs. 2 and 3) the nucleolar material is readily observed under phase microscopy. However, in preparations fixed in a 1:1 acetic:lactic acid mixture, the nucleolus is no longer observable. In such preparations, as illustrated in Fig. 4, we found that the spheres, usually in relatively small numbers, were localized near the heterochromatin material at the base of the X chromosome. This suggests the presence of rDNA radiating out from this region.

Control experiments were performed in which the hybridization was carried out with rRNA which was not coupled to biotin. These preparations showed no localization of spheres over the nucleolus or any other part of the chromosomes.

As described in Methods (p. 000), the incubation with avidin-spheres is carried out in the presence of 1 mg/ml of cytochrome c. If cytochrome c is not used, there is a high level of nonspecific attachment of spheres all over the slides. Avidin is a very positive protein with an isoelectric point of ca. 10.5 (Melamed and Green, 1963). Presumably the positively charged cytochrome c competes for negatively charged sites on the slides and thus decreases the amount of nonspecific labeling.

In the procedure described, we observed no localization of spheres on any region of the chromosome other than that containing nucleolar material. But we have observed on some slides that there are areas which contain no chromosome structures but are heavily labeled with spheres. These dense areas were observed using both rRNA-biotin and rRNA without biotin. The cause of this occasional dense labeling is not known. However, its occurrence is infrequent and does not pose a significant problem for the identification of reiterated genes such as the ribosomal genes.

Discussion

In developing a sphere labeling procedure, we have been guided by the philosophy that the polynucleotide probe (in this case rRNA) should be only mildly modified, so that steric hindrance in the slow hybridization reaction is minimal. The massive sphere label then attaches by a reaction between biotin and avidin, which is rapid and complete for the free molecules.

The results reported here show that the polymer sphere labeling procedure and SEM observation is effective for mapping the 200-fold reiterated (Tartof

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ome. ca. ×15,000

and Perry, 1970) rRNA genes in the polytene chromosomes of *Drosophila melanogaster*. In principle, the method should give higher resolution than autoradiography at the optical microscope level. It is in some ways more convenient than autoradiography because long exposure times are avoided. It can be applied to any polynucleotide and does not require high specific activity radioactive labeling.

The DNA in the nucleolus appears to be well dispersed and may be especially readily available for hybridization and for the subsequent penetration and reaction of avidin-spheres. Further tests will be carried out to determine the effectiveness of the procedure for reiterated genes occurring in other regions of the chromosome, for single-copy genes in polytene chromosomes, and for gene mapping in nonpolytene chromosomes.

Acknowledgments. J.E.M., N.D.H., T.R.B., and M.P. are recipients of fellowships from the Jane Coffin Childs Foundation, the National Institute of General Medical Sciences, the Helen Hay Whitney Foundation, and the American Cancer Society, respectively. Drs. Lynne Angerer and Pauline Yen have contributed to the general developments of techniques for biotin-avidin labeling. This research has been supported by research grant GB 23343 from the National Science Foundation to H.K.M., and by research grant GM 10991 from the National Institute of General Medical Sciences to N.D.

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Received June 20—July 27, 1975 / Accepted July 15, 1975 by J. G. Gall
Ready for press August 8, 1975

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COUPLING OF ENZYMES TO PROTEINS WITH GLUTARALDEHYDE.

USE OF THE CONJUGATES FOR THE DETECTION OF ANTIGENS AND ANTIBODIES

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(*Received* 30 *April* 1968)

Abstract—Conjugation of peroxidase, glucose oxidase, tyrosinase and alkaline phosphatase to human immunoglobulin-*G*, human serum albumin, sheep antibody and rabbit antibody was carried out with glutaraldehyde. The conjugates retained a substantial part of their immunological and enzymatic activity. They were utilized for the intracellular detection of antigens and antibodies and they were employed for the characterization of the antibodies after immunoelectrophoresis.

INTRODUCTION

Antigens and antibodies labeled with fluorescent or radioactive substances have been used for the detection of specific immune complexes[1–5]. Recently with the aid of water soluble carbodiimides[6], cyanuric chloride[7] or *p*,*p*′ difluoro-*m*-*m*′ dinitrophenyl sulfone[8, 9] enzymes were coupled to antigens and antibodies. The catalytic activity of these enzyme-labeled proteins, which was detectable by histochemical methods, has been used either for the localisation of antigens[8, 9] and antibodies[7, 10] within cells or for the characterization of antibodies after immunoelectrophoresis[6, 7].

In the present paper, we report the conjugation of enzymes with proteins using glutaraldehyde as the coupling agent and the use of the conjugates obtained (a) for the detection of antigens or antibodies in cell preparations and (b) for the characterization of the antibodies after immunoelectrophoresis.

MATERIALS AND METHODS

Materials

Human γ-globulins were obtained from the Netherland Red Cross and rabbit γ-globulins from Pentex (Kankakee, Illinois). Horse radish peroxidase RZ 3·0 or RZ 0·6 and 25 per cent aqueous solution of glutaraldehyde were products of Schuchardt, München. Horse radish peroxidase (RZ 3·0) and glucose oxidase (90 units/mg) of 'analytical reagent grade', were purchased from C. F. Boehringer, Mannheim.

Purified E. Coli alkaline phosphatase (BAPSF 10 units/mg), chicken intestinal alkaline phosphatase (PC 0·9 units/mg) and mushroom tyrosinase (TY 500 units/mg) were obtained from Worthington, New Jersey. Purified aspergillus niger glucose oxidase (type II 13–18 units/mg) and all the other chemicals were purchased from Sigma Co. St. Louis.

Antisera

The rabbit antisera to peroxidase, glucose oxidase and alkaline phosphatase were prepared according to the following procedure. One year old rabbits received the first day 12–15 intradermal injections at the dorsal region of a total of 5 mg of protein in 0·7 ml of 0·15 M NaCl and 0·3 ml of complete Freund adjuvant This operation was repeated on the 15th and 30th day and the animals were bled the 37th day by cannulating the internal carotid artery. The rabbit antiserum to human IgG was obtained as described in the following paper[11]. Sheep serum anti-rabbit γ-globulins and horse serum anti-whole rabbit serum were obtained from the Institut Pasteur, Paris.

Concentration of proteins

Protein content was determined by a modified biuret method[12]. Peroxidase content of different preparations was obtained from optical density at 403 mμ and that of glucose oxidase at 460 mμ.

Immunoelectrophoretic analysis

Immunoelectrophoresis was carried out according to the method of Grabar and Williams[13] as modified by Scheidegger[14].

Electrophoresis in acrylamide-agarose gels

Electrophoresis in acrylamide-agarose gels was carried out according to a method previously described[15] on glass plates, 10 × 5 cm. A gel of 5 per cent in acrylamide and 0·8 per cent in agarose was used.

Molecular sieving chromatography

This was carried out on a chromatographic column packed with Sephadex G-200 (45 × 1 cm) and equilibrated with 0·1 M phosphate buffer pH 6·8.

Ultracentrifugation analysis

Ultracentrifugation analysis was performed with a Spinco Model *E* analytical ultracentrifuge. The analysis of the different protein preparations was carried out in 0·1 M phosphate buffer pH 6·8 at 59,780 rev/min and 20°. The values of *S* given in this work were not corrected for protein and salt concentration.

Isolation of antibodies

Rabbit antibody anti-human IgG and sheep antibody anti-rabbit γ-globulins were isolated by passage of the corresponding whole antisera on the homologous insolubilized antigen using procedures described in detail elsewhere[11]. Isolated antibody if necessary was concentrated on saccharose powder or by pervaporation and then dialyzed against phosphate buffer 0·1 M pH 6·8. The final antibody content was 5–10 mg of protein per ml.

Immunofluorescence microscopy

Labeling of antibody or γ-globulins fraction of antiserum with fluorescein isothiocyanate and examination of cellular preparations under fluorescence microscopy were carried out following procedures described in detail elsewhere[16].

Histochemical detection of enzymes

Histochemical coloration of enzymes in cellular preparations was carried out according to methods described in detail elsewhere[17, 18]. Peroxidase activity was revealed using either the NADI (p. 900 of reference 17) or the 3·3′ diaminobenzidine[19] reagents, but in this last case post incubation in osmium tetroxide was omitted.

Alkaline or acid phosphatase activity was detected by the Naphthol-AS technic (p. 275–276 of reference 18).

Tyrosinase activity was revealed by employing dihydroxyphenylalanine as substrate (p. 458 of reference 18).

Glucose oxidase activity was localized by incubating the preparation in the absence of light in 20 ml of 0·1 M phosphate buffer pH 6·9–7·0 containing 150 mg of *D*-glucose, 10 mg MTT* and 2 mg of phenazine methasulfate. The preparation was then washed for 3–5 min in phosphate buffer and then rinsed with distilled H_2O.

Detection of enzymes on immunoelectrophoretic slides was carried out on slides dried under filter paper, using procedures already described[20] or by utilizing one of the above mentioned technics. After coloration the plates were washed in some changes of distilled H_2O and then dried.

EXPERIMENTAL RESULTS AND DISCUSSION

Coupling of enzymes to proteins with the aid of glutaraldehyde

To couple an enzyme with a protein a bi- or multifunctional reagent is needed. This reagent should be specially well selected when an enzyme has to be coupled to an antigen or to an antibody with the view to obtain a protein-enzyme complex which retains a significant part of its immunological and enzymatic activity. Of several such coupling reagents which were assayed, only cyanuric chloride gave satisfactory results, but these were irreproducible when this reagent was assayed for the labeling of antibody[7, 10].

Glutaraldehyde was then tried as it has been reported that intracellular enzymatic activities were well preserved in cellular preparations which were fixed with this reagent[21] and because it was found that antigenic determinants or antibody activity was partially preserved when glutaraldehyde was used for the insolubilization of antigen or antibody[11]. The results obtained have shown that in fact glutaraldehyde was by far the most effective and suitable reagent for producing enzyme-protein complexes which retained a part of their enzymatic and immunological specificity.

The coupling of the sheep antibody anti-rabbit γ-globulins with peroxidase is given as an example of the procedure which is currently used to link enzyme to proteins with the aid of glutaraldehyde. To 1 ml of 0·1 M phosphate buffer, pH 6·8 containing 5 mg of antibody 12 mg of peroxidase (RZ 3) are dissolved. While the solution is gently stirred, 0·05 ml of a 1 per cent aqueous solution of glutaraldehyde is added dropwise. The reaction mixture is allowed to stand for 2 hr at room temperature and then dialyzed against two changes of 5 liters

*MTT = (3-(4·5-dimethyl-thiazolyl)-2·5 diphenylmonotetrazolium bromide); usually named thiozyl-Blue.

of buffered physiological saline (PBS) at 4°C overnight. The precipitate formed is removed by centrifugation for 30 min at +4°C and 20,000 rev/min in rotor 40 of a Spinco ultracentrifuge. This stock solution of peroxidase labeled-antibody is kept at +4°C until used. It could be used for at least three months without noting any appreciable loss in its catalytic and immunological specificities. Coupling of glucose oxidase, alkaline phosphatase and tyrosinase with human, rabbit and sheep γ-globulins or purified antibody is carried out with the same general procedure.

In Table 1 are reported the quantities in enzyme, protein and glutaraldehyde required for several protein-enzyme systems in order to have an efficient coupling and to obtain protein-enzyme complexes which retain both their immunological specificity and enzymatic activity.

Table 1. Quantities of reactants giving protein-enzyme complexes immunologically and enzymatically active

Protein	Quantity (mg)	Enzyme*	Quantity (mg)	Volume† of enzyme-protein solution before the addition of glutaraldehyde (ml)	Quantity‡ of glutaraldehyde added (ml)
Human IgG	5	Peroxidase	12	1	0·05
,, ,,	5	Phosphatase	10	1·3	0·10
,, ,,	12	Phosphatase[a]	50	2	0·40
,, ,,	12	Glucose oxidase[b]	50	2	0·40
Human serum albumin	5	Glucose oxidase[b]	50	1	0·80
Glucose oxidase[b]	5	Phosphatase	10	1·3	0·15
Sheep anti-rabbit γ-globulins	5	Phosphatase	10	2	0·15
,, ,, ,,	5	Glucose oxidase	10	1	0·15
,, ,, ,,	5	Tyrosinase	25	1	0·15
,, ,, ,	5	Peroxidase	12	1	0·05
Rabbit anti-human IgG	5	Peroxidase	12	1	0·05

*The different enzyme preparations employed were: peroxidase = peroxidase RZ 3; phosphatase = alkaline phosphatase BAPSF 10 units/mg; phosphatase[a] = alkaline phosphatase PC 0·9 units/mg; glucose oxidase = glucose oxidase 90 units/mg 'analytical reagent grade'; glucose oxidase[b] = crude preparation of aspergillus niger glucose oxidase type II; Tyrosinase: mushroom tyrosinase TY 500 units/mg.

†Phosphate buffer 0·1 M pH 6·8.

‡1% (w/v) of aqueous solution of glutaraldehyde.

The information presently available seems to indicate that most often free amino groups do not actively participate in the catalytic activity of an enzyme molecule, and the same seems to hold true for antibody activity. The observation that with glutaraldehyde it is almost exclusively free amino groups of proteins which participate in the cross-linking reaction might explain the

ccessful results obtained (for the specificity of glutaraldehyde see discussion reference [11]).

To test that after the coupling reaction an efficient linking between protein d enzyme has been achieved, the preparation of protein and enzyme before d after the addition of glutaraldehyde is subjected to immunolelectrophoresis d bands are developed with anti-protein and anti-enzyme antisera. The im-unoelectrophoretic slides are washed in PBS for 4 days, dried and some of em are stained for protein while the others are revealed for enzymatic activity. n general, it was observed that after coupling the immunoelectrophoretic and which was revealed with the anti-protein antiserum possessed a strong nzymatic activity while no such an activity was visible before the coupling reaction.

The enzyme labeled protein was also tested for its capacity to detect, after mmunoelectrophoresis of a known immune serum, homologous antiprotein ntibody, according to the procedure to be described (see : characterization of ntibody after immunoelectrophoresis with enzyme labeled antigens). Using his test an efficient labeling of proteins was noted with all the enzyme protein ystems assayed and in which glutaraldehyde was utilized as the coupling agent. The effectiveness of the glutaraldehyde coupling was particularly well illus-rated when the protein to be labeled by an enzyme possessed also a catalytic ctivity (Fig. 1).

Experiments were carried out using several techniques in order to determine he nature of the complexes which were produced after the coupling reaction. mmunoelectrophoresis (or double diffusion) of the enzyme labeled protein howed immunodiffusion pictures of high heterogeneity. Enzyme and protein fter coupling were found to possess higher electrophoretic mobilities. Electro-horesis of the enzyme labeled protein on acrylamide agarose gels has not shown istinct electrophoretic bands but rather a continuous protein pattern having a ide range of electrophoretic mobilities and possessing enzymatic activity. hromatographic runs on Sephadex G-200 were performed using rabbit IgG oupled either with peroxidase or with glucose oxidase. In both cases, the IgG nzyme complexes were chromatographed with the void volume of the column. Ultracentrifugation analyses were carried out using rabbit IgG and different nzymes in order to determine the sedimentation coefficients of the prepara-ons before and after the coupling reaction. The analyses of the reaction mix-re have shown an irregular ultracentrifugal base line and the presence of veral fast sedimenting small peaks. In addition, these experiments revealed at, in the reaction mixture, no protein peak possessing the sedimentation efficient of an IgG was present while peaks having almost the same sedimenta-n coefficient as the initial enzyme preparation were present (Table 2). These servations would probably mean that, in the reaction mixture, highly hetero-neous populations of IgG-enzyme complexes, not detectable by ultracentri-gation analyses are present. The relative quantity of the enzyme in the solu-ons can be calculated from the ultracentrifugation area peak. Then, if the antity of the enzyme remaining after the coupling reaction is subtracted from initial quantity of the enzyme, one can deduce that the average ratio of nzyme to IgG in the complexes varies between one and two.

Table 2. Sedimentation coefficients of rabbit IgG and enzymes before and after the coupling reaction*

IgG and enzyme	Sedimentation coefficient (*S*) before coupling	Sedimentation coefficient (*S*) after coupling
IgG + peroxidase	6·25 3·13	3·38
IgG + glucose oxidase	6·25 4·24–6·87–9·3	4·72–7·58–10·12
IgG + alkaline phosphatase	6·25 1·3–5·58	2·09–6·2

*Coupling of IgG to peroxidase (RZ3), phosphatase (10 units/mg) and glucose oxidase (90 units/mg) were carried out under the conditions reported in Table 1.

From all the above cited evidences, it can be concluded that cross-linking of enzymes to proteins with glutaraldehyde produces a heterogeneous population of enzymatically and immunologically active complexes.

Characterization of antibody after immunoelectrophoresis with enzyme labeled antigens

The same principle already described for radio-immunoelectrophoresis[4] was used to characterize the antibodies present in antisera after immunoelectrophoresis. The sera from normal and immunized animals are subjected to immunoelectrophoresis and bands are developed with a suitable antiserum. The immunoelectrophoretic slides are washed for 3 days in PBS . The slides are then immersed overnight at room temperature in a solution of PBS containing the enzyme-labeled antigen in a final concentration of 0·1–0·3 mg of antigen per ml. The slides are then washed again for 4 days and dried under filter paper. The enzyme-labeled antigen fixed to the homologous antigen antibody precipitate is then revealed by incubating the slides in the appropriate enzyme substrates. Colored arcs reveal the presence of antibody (Figs. 2, 3).

Agarose gel is a more neutral substance than agar gel and thus the excess of the enzyme-antigen complexes are more readily washed out. Therefore immunoelectrophoresis in agarose gel has to be prefered when enzyme labeled antigen is used for the detection of antibodies.

Most often peroxidase, glucose oxidase, and alkaline or acid phosphatases were used as markers of the antigen. With all these enzymes satisfactory results were obtained but it should be stressed that normal sera have to be run in parallel because some serum constituents which do not belong to the immunoglobulin family possess enzymatic activities. When possible, labeling with glucose oxidase was prefered because no such enzyme was detected in the immunoelectrophoretic slides of all the normal sera we have examined.

For the characterization of antibodies in gel diffusion media highly purified enzyme preparations are not necessary for the labeling of antigen. Partially

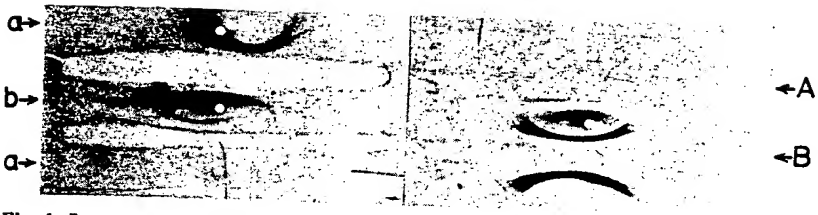

Fig. 1. Immunoelectrophoresis of a solution containing glucose oxidase and alkaline phosphatase before and after reaction with glutaraldehyde. a: solution before coupling, b: solution after coupling, A: rabbit antiserum anti-phosphatase, B: rabbit antiserum anti-glucose oxidase. Left plate: stained for phosphatase activity. Right plate: stained for glucose oxidase activity. Note that after the coupling the band which is developed with anti-glucose oxidase antiserum possess phosphatase activity and that developed with anti-phosphatase possess glucose oxidase activity.

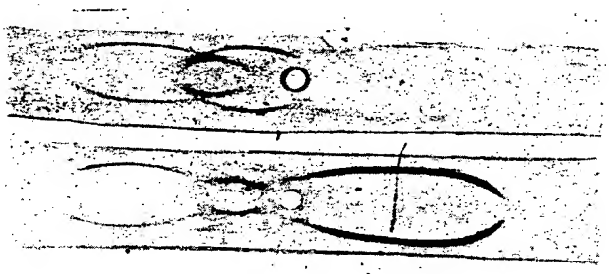

Fig. 2. Characterization of rabbit antibody to human serum albumin. Rabbit sera developed with horse anti-whole rabbit serum. Incubation in a solution of peroxidase labeled albumin and revealed with NADI reagent. Top: normal serum. Bottom: immunserum.

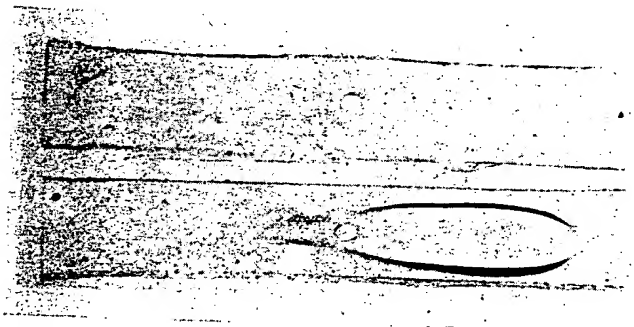

Fig. 3. Characterization of rabbit antibody to human-IgG. Rabbit sera developed with horse anti-whole rabbit serum. Incubation in a solution of glucose oxidase labeled IgG and revealed with MTT reagent. Top: normal serum. Bottom: immunserum.

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Fig. 4. Intracellular detection of rabbit γ-globulins. Spleen cell suspension of an hyperimmunized rabbit. Incubation in a solution of peroxidase labeled sheep antibody anti-rabbit γ-globulins and revealed with diaminobenzidine reagent.

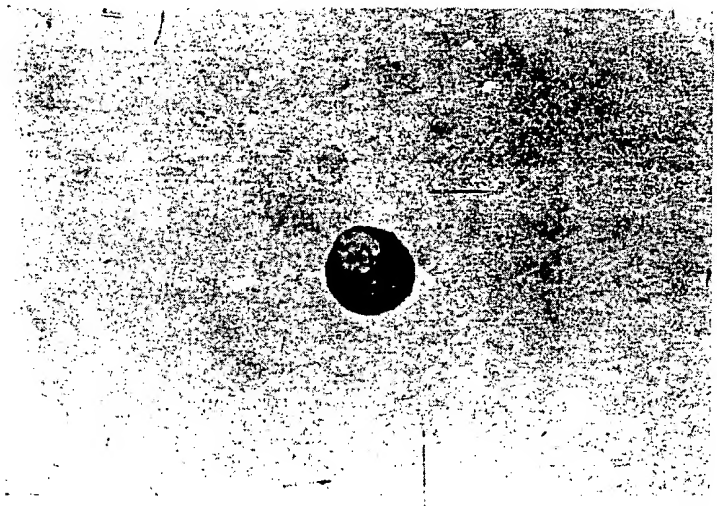

Fig. 5. Intracellular detection of rabbit γ-globulins. Spleen cell suspension of an hyperimmunized rabbit. Incubation in a solution of glucose oxidase labeled sheep antibody anti-rabbit γ-globulins and revealed with MTT reagent.

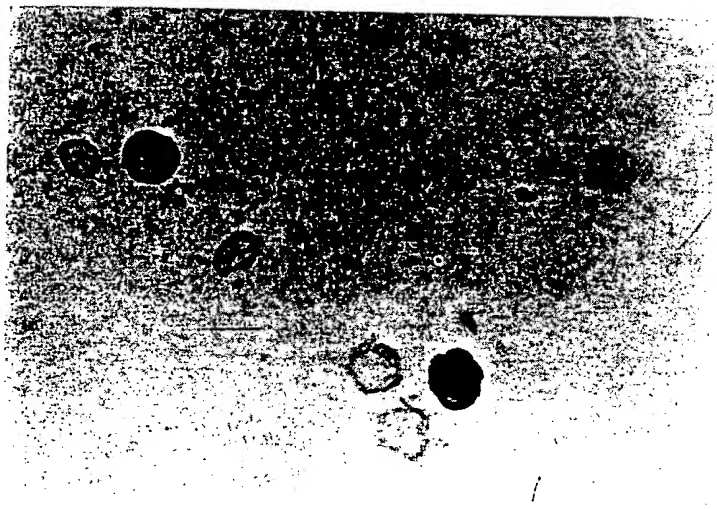

Fig. 6. Intracellular detection of rabbit anti-human IgG antibody. Spleen cell suspension of a rabbit immunized with human IgG. Incubation in a solution of alkaline phosphatase labeled human IgG and revealed with Naphthol AS technic.

urified preparations of peroxidase (RZ 0·6), tyrosinase, alkaline or acid phosatase, glucose oxidase and other enzymes can be employed. Even if the ecific activity of the labeled antigen is low, the relatively high quantity of tigen that the antibody can fix would be sufficient to reveal the enzymatic tivity. However, one has to bear in mind that to detect minute amounts of antiody purified enzymes are indispensable.

Using enzyme-labeled antigens, rabbit, mouse, goat, sheep and horse antiodies were characterized after immunoelectrophoresis. The same principle n also be applied for the detection of anti-hapten antibodies if an enzyme is sed as the protein carrier of the hapten [6].

No direct comparison could be made on the relative sensitivity for the detecon of antibody in gel diffusion media, by using in parallel an enzyme labeled nd a radioisotope target antigen. However, it would appear that some advanages exist when an enzyme-labeled antigen is used. Thus:

a) the enzymatic activity of the background is negligible;

b) the whole procedure is less time consuming;

c) direct documents on the immunoelectrophoretic slides are obtained;

d) by selecting the enzymes, two or more different antibodies might be characrized on the same plate. Thus for example, peroxidase labeled IgG and phosatase labeled albumin can be utilized to detect on the same electrophoretic ides anti-IgG and anti-albumin antibodies. The procedure employed would be e same as those reported later for the detection of two intracellular conituents.

Detection of intracellular constituents with enzyme-labeled proteins

It should be emphasized that, in early experiments, enzyme-labeled antibody epared with a variety of coupling agents and enzymes often failed to detect e antigens. This difficulty was overcome when:

purified antibody was used;

the purest commercially available enzymes were utilized as markers and

the coupling reaction was carried out with the aid of glutaraldehyde. Taking o account these three conditions, successful and reproducible results were tained.

The detection of rabbit γ-globulins in the splenic cells of this animal, by ng a peroxidase labeled sheep antibody anti-rabbit γ-globulins, is given n example of the procedure which is currently used to detect intracellular nstituents.

Frozen sections from rabbit spleen were cut and suspensions of cells were ted on microscopic slides. The spleen preparations were fixed for 45 min at om temperature in a mixture of 60 volumes of alcohol and 40 volumes of ether, shed for 15 min in PBS and then stored at +4°C. To detect the cells containing γ-globulins, the preparations were incubated for 3 hr at room temperature 0·1 M phosphate buffer pH 7·2 containing the peroxidase-labeled antibody at nal concentration of 0·5 mg of antibody per ml (stock solution of peroxidase eled antibody diluted 10 times). The slides were then rinsed twice, for 3 min n time, in PBS, incubated with NADI or diaminobenzidine reagent for 5–10 n, rinsed with distilled H_2O, and examined under the light microscope

(Fig. 4). Eventually, the preparation after rinsing was dehydrated, cleared and mounted in a suitable natural or synthetic medium[17, 18]. The detection of rabbit γ-globulins in spleen cells by sheep antibody labeled with either glucose oxidase, alkaline phosphatase or tyrosinase is carried out in the same general way (Fig. 5). For revealing glucose oxidase and phosphatase activity, most often incubation times of 15–30 min were used, while 2 hr was found to be suitable for tyrosinase activity. In any case, it is possible to remove the cellular preparation from the incubation mixture, examine this under the light microscope, and if the staining is faint, reincubate the preparation with the substrate. In addition, when the quantity of the antigen present in the cell is quite small, one can incubate the cellular preparations for longer periods of time, taking the precaution however to replace from time to time the incubation mixture with a fresh one, and so increase the possibility of detecting the antigen.

Each spleen preparation under investigation was incubated in a solution of an unrelated antibody solution coupled with the enzyme and in a solution containing the enzyme alone. On the other hand, preparations from normal animals were incubated with the enzyme-labeled antibody. All the controls were negative, except for endogenous enzyme activities. Thus, erythrocytes were colored when peroxidase was detected while polynuclear cells were colored when alkaline phosphatase or tyrosinase was detected. No coloration on control cell preparations was observed when glucose oxidase was detected.

To localize antibody in immunocompetent cells, the same general procedure as described above was utilized. The cell preparations were fixed* as before and then incubated for 4 hr at room temperature in a solution of 0·1 M phosphate buffer pH 7·2 containing the enzyme-labeled antigen in a final concentration of 0·3–0·5 mg of antigen/ml. The cell preparations were then treated in exactly the same way as for the detection of intracellular antigen by enzyme-labeled antibody (Fig. 6).

In almost all experiments, spleen cells from hyperimmunized animals were prefered because, in these preparations, at least twenty times more cells containing γ-globulins were present than in preparations from normal animals.

Many enzymes for which a histochemical reaction exists[17, 18], can be used as markers for the labeling of antigen or antibody. In addition two (or more) adequately chosen enzymes can be used for the labeling of two different proteins and these enzyme-labeled preparations can be utilized for the detection of two different intracellular constituents. Thus, for example, rabbits were hyperimmunized with human IgG and preparations of spleen cells were incubated in a solution of IgG labeled with peroxidase, washed, and then incubated in a solution, labeled with phosphatase, of sheep antibody anti-rabbit γ-globulins. After washing, the plates were revealed for peroxidase activity with 3·3′ diaminobenzidine reagent and examined under microscope. The plates were then colored for phosphatase activity and reexamined. Cells showing only phosphatase activity were detected but all the cells having peroxidase activity possessed also phosphatase activity. Probably this would mean that, at the same time, anti-

*Besides alcohol-ether, other fixatives like methyl alcohol, acetone and buffered solutions of formaldehyde were assayed. All these fixatives gave satisfactory results either for antigen or for antibody detection.

uman IgG antibody and normal rabbit γ-globulins or/and antibodies of pecificities other than IgG were present in immunocompetent cells of rabbit yperimmunized with human IgG.

Experiments were carried out to determine the relative sensitivity of the nzyme-labeled antibodies as opposed to the fluorescein labeled antibody echnic. Peroxidase-labeled and fluorescein-labeled sheep antibody anti-abbit γ-globulins were tested in parallel with a spleen cell suspension from n hyperimmunized rabbit. The relative number of cells counted with peroxi-ase-labeled antibody was found to be twice as high as that of the fluorescein-abeled antibody (20 counts, in each count about 100 cells were examined). ssentially the same number of positive cells were counted with either phos-hatase, glucose oxidase or fluorescein-labeled antibody. Although no quan-itative data were obtained with tyrosinase labeled antibody, it seemed that the ensitivity of this preparation was less than with the other enzyme-labeled ntibody preparations. Almost the same results as those reported here were btained when fluorescein or enzyme-labeled antibodies were compared for heir sensitivity to detect virus antigens in cell infected cultures [22]. In addition, xperiments in progress in this laboratory using antibodies of different speci-icities, labeled either with various enzymes or with fluorescein, tend to show that n a general way, for the detection of intracellular constituents, enzyme labeled ntibodies are at least as sensitive as fluorescein labeled antibodies.

Enzyme labeled antibody, when it is used for the detection of antigen, has everal advantages and disadvantages when it is compared to the fluorescein abeled antibody. Thus, in general, one has the impression that the background s less with the enzyme than with the fluorescein-labeled antibody. The fact hat pure antibody is indispensable to prepare an effective enzyme-labeled ntibody makes the whole procedure for the detection of an antigen more aborious and time consuming than with a fluorescein-labeled γ-globulin anti-erum fraction. However, examination of cellular preparations under the light icroscope is less tedious than examination under the fluorescence microscope. ommercially available preparations of pure enzymes are more costly than uorescein reagents but, on the contrary, an expensive fluorescence micro-cope is not needed when enzyme-labeled antibody is used. Enzyme-labeled ntibody has the advantage over fluorescein and ferritin-labeled antibody [23] hat the same preparation can be utilized both for light and electron micro-copic studies [8, 9, 10]. Intracellular proteins seem to be well localized when uorescein is used as a marker. An equally good localization is obtained with nzymes and this was particularly well illustrated by electron microscopic studies sing either peroxidase or alkaline phosphatase for the detection of antibody rmation [24, 25].

nowledgement—The author would like to acknowledge Miss Micheline Faivre for erforming the ultracentifugation analyses.

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